BIOCHEMICAL STUDIES ON BASOPHIL LEUCOCYTES

by

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TO RUTH

Declaration

I confirm that the work in this thesis was conceived, planned and either executed by myself and/or by a technical assistant working directly under my supervision.

Date ...24th...May..1979.....

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SUMMARY

Several variables associated with the assimilation of histamine by the guinea pig basophil have been studied. The experimental model has been an *in vitro* technique which measures the uptake of radiolabelled histidine and histamine, and the synthesis of new histamine from the isotopic histidine incorporated. The cell preparations used were prepared by modifications of previously published procedures for obtaining large numbers of basophils from the bone marrow of the guinea pig.

Using this technique it was found that only the guinea pig basophil, when compared to eosinophils, neutrophils and mononuclear cells, incorporated appreciable amounts of ^{14}C-histidine and converted it to new histamine. In contrast, histamine was taken up by all these cell types, but in very small concentrations. Histidine uptake and the amount of histamine formed *de novo* was directly related to the number of basophils, the time of incubation and the substrate concentration. In addition, the uptake of radiolabelled histidine was dependent on the integrity of a number of biochemical pathways. Thus agents which inhibited glycolysis, oxidative phosphorylation, protein synthesis and RNA synthesis decreased the amount of ^{14}C-histidine incorporated in a dose-dependent fashion. In contrast, conversion of isotopic histidine to histamine within the cell was not affected by these agents. This part of the study suggested that histidine is preferentially incorporated into the basophil by an active mediated process but that once the histidine is

taken up these requirements do not apply to the formation of new histamine. In contrast, histamine appears to enter the cells by passive mediated transport and in relatively small amounts.

Histamine and the prostaglandins, PGE_1, PGE_2 and $PGF_{2\alpha}$ - pharmacological mediators known to be released during type I hypersensitivity reactions in the guinea pig - increased the amounts of ^{14}C-histidine incorporated and ^{14}C-histamine formed de novo. By the use of histamine receptor antagonists the enhancement by histamine was shown to involve H1 receptors. The histamine metabolites, imidazoleacetic acid and 1,4-methylimidazoleacetic acid, were also capable of increasing ^{14}C-histidine uptake and ^{14}C-histamine formation, although to a lesser extent than histamine. However, a number of other pharmacological mediators, including bradykinin, 5-hydroxytryptamine, and the human ECF-A tetrapeptides (Val-Gly-Ser-Glu and Ala-Gly-Ser-Glu), several anti-inflammatory drugs and the anti-allergy agent, disodium cromoglycate, were without effect. Apart from histamine and some of its major catabolites all these agents had no effect on ^{14}C-histamine uptake. Thus histamine and prostaglandins may play a bioregulatory role in the events accompanying the uptake of histidine by histamine forming cells.

When actively sensitized basophils were challenged with specific antigen they were still capable of incorporating ^{14}C-histidine and forming ^{14}C-histamine de novo. Indeed, there was a time-dependent increase in ^{14}C-histidine uptake and ^{14}C-histamine formation by these challenged cells when compared to unchallenged controls. Thus the release of

histamine and the presence of extracellular histamine and prostaglandins might stimulate basophils to replenish their stored histamine by increased amine formation de novo.

Studies on the effects of eosinophils on histamine replenishment were also performed. Eosinophils had no effect on either the amount of ^{14}C-histidine incorporated or ^{14}C-histamine formed by antigen stimulated or unchallenged basophils. Therefore, using this in vitro model the eosinophil was apparently unable to influence basophil histamine replenishment.

In summary, this study has attempted to provide insight into the mechanisms of histamine assimilation within the guinea pig basophil and the factors which influence its regulation.

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CHAPTER I - INTRODUCTION

SECTION I - IMMEDIATE-TYPE (TYPE I) HYPERSENSITIVITY

1.0 INTRODUCTION

Mast cells and basophils were first described at the end of the nineteenth century (reviewed by Michels, 1938) although it was not until 1941 that the first reference to mast cell damage in anaphylaxis was reported (Jaques and Waters, 1941). The concept that a membrane receptor might play a role in anaphylactic reactions was, however, proposed by Dale (1913) and Weil (1913) long before the role of mast cells in this reaction or the nature of the immunoglobulin involved was appreciated.

The fact that allergic serum contained a humoral factor which upon passive transfer may cause allergic symptoms in a healthy individual was first observed by Ramirez in 1919 and further extended by the classical experiments of Prausnity and Küstner in 1921 (reviewed by Prausnity, 1955). The studies of Ishizaka and Ishizaka during the 1960's clearly established that immunoglobulin E (IgE) was the reagin implicated in the pathogenesis of atopic allergy (reviewed by Ishizaka and Ishizaka, 1975).

The first observations of antigen-induced degranulation or histamine release from isolated mast cells and basophils was reported at the end of the 1950's (reviewed by Mongar and Schild, 1962). Since then the molecular basis of histamine storage, the presence and structure of various other pharmacological mediators and the biochemical events associated with their release have been determined in a variety of species.

2.0 PRIMARY TARGET CELLS

Tissue mast cells develop from undifferentiated mononuclear mesenchymal cells in the perivascular areas of connective tissue (Asboe-Hansen, 1973). They are ubiquitous in the loose connective tissues of higher vertebrates and, in general, are distributed along blood vessels. Mast cells, along with the basophil leucocyte (see Section II) have the capacity to bind IgE on to their surface. Subsequent interaction between specific antigen and bound IgE results in degranulation with mediator release.

3.0 IMMUNOGLOBULINS ASSOCIATED WITH MAST CELL AND BASOPHIL DEGRANULATION

Immunoglobulin molecules capable of interacting with homologous or closely related cell membrane receptors and generating a trigger for antigen-induced noncytotoxic, complement-independent release of intracellular granular constituents such as histamine are functionally defined as homocytotropic (Becker and Austen, 1966).

The IgE class of antibody which has been studied extensively in man and is found in many other species, fits this definition. However, recently certain immunoglobulin subclasses have been described in several species which possess the same biological properties.

3.1 Immunoglobulin E

In man, IgE is a glycoprotein of molecular weight 188,000 containing 12% carbohydrate. Electrophoretically it migrates as a γ_1 globulin (Johansson and Bennich, 1967), circulates as a minor component of normal blood, 0.1 to 0.4 $\mu g.ml^{-1}$, and like all other immunoglobulins it is produced by plasma cells. IgE is composed of two light and two heavy chains and is probably divalent. It has a short half life in the serum of about 2-3 days (Waldmann, 1969); by contrast $T\frac{1}{2}$ of IgG is 25 days. It does not "fix complement" via the classical pathway when reacting with antigen but can activate the alternative pathway (Ishizaka and Ishizaka, 1975).

The fixation of IgE to human basophils has been demonstrated by autoradiography and electron microscopy (Ishizaka *et al*, 1970a; Sullivan *et al*, 1971). The

functional presence of IgE, i.e. to release histamine, constitutes indirect evidence for the existence of a receptor. This approach has been used to show that binding occurs via the Fc portion of the molecule (Ishizaka et al, 1970b) and that it is this part which is heat labile. IgE is distributed over the entire basophil surface, as judged by immunofluorescence and immunoferritin electron microscopy, but can be redistributed with anti-IgE to form patches or "caps" in a manner analogous to "capping" observed with lymphocytes and other cell types (Taylor et al, 1971).

The affinity of IgE for the cell surface is normally very high at physiological pH, of the order 10^{-8} to 10^{-9} moles.l^{-1} for human basophils (Ishizaka et al, 1973). Normally each cell is thought to possess about 10-40 x 10^3 molecules of bound IgE but this may be increased three- to seven-fold by exposure to high concentrations of IgE. It has been estimated that there are 30-100 x 10^3 receptor sites for IgE on a normal basophil and that in an atopic individual the vast majority may be filled. The binding between IgE and the cell surface is reversible and does not involve a covalent bond (Ishizaka and Ishizaka, 1974; Ishizaka et al, 1973).

3.2 Non-IgE homocytotropic antibodies

Reagin IgE antibodies of man and IgE-like antibodies of animals normally occur in very small amounts in normal serum, are heat labile and can confer passive sensitization for several weeks. Some species have anaphylactic antibodies of two immunoglobulin classes, IgE and a subclass of IgG.

The non-IgE antibodies have certain characteristics in common. They have a gamma electrophoretic mobility, belong to an immunoglobulin class present in mg amounts in serum, have a sedimentation coefficient of 7S and have a relatively rapid turnover (Stechschulte, 1978). In contrast to IgE they are relatively heat stable, resistant to sulphydryl reagents and involve complement in selected reactions.

Rat IgGa, mouse IgG_1, sheep IgS, guinea pig IgG_1 and rabbit IgGa have all been identified as homocytotropic antibodies (Stechschulte, 1978; Esteves et al, 1974). Recently IgG_4 has been implicated as an anaphylactic IgG antibody in man (van der Giessein et al, 1976; Shakib et al, 1977)

4.0 BIOCHEMICAL MECHANISMS OF MEDIATOR RELEASE

The interaction of fixed tissue mast cells or circulating basophils with a variety of stimuli results in the secretion of a number of mediators which play a prominent role in the inflammatory process.

4.1 Immunoglobulins in mediated secretion

Of the wide variety of stimuli known to initiate secretion from mast cells, the reaction of cell-bound IgE with antigen has major biological relevance. It has been demonstrated to occur *in vitro* and *in vivo* and represents an integral component of acute allergic reactions.

It appears that the initial signal for mediator release is generated by the cross-linking of surface IgE by antigen (Ishizaka and Ishizaka, 1975). Studies in a variety of *in vitro* model systems have revealed that the release process is non-cytotoxic and essentially a selective secretion. The antigen-induced release of histamine from IgE-sensitized human basophils occurs without loss of intracellular K^+ ions (Lichtenstein and Osler, 1966), impairment of vital dye exclusion (Lichtenstein and Osler, 1964) or loss of motility under phase contrast microscopy (Hastie, 1971).

The mechanism of anaphylactic histamine release from mast cells was first investigated by Mongar and Schild (1957a; 1957b; 1958) using actively sensitized guinea pig lung fragments. Later the biochemical reactions involved were also studied in the mast cells from other species including rats (Uvnas and Thon, 1961), mice (Prouvost-Danon *et al*, 1966) and man (Kaliner and Austen, 1973) and in human, rabbit and

guinea pig basophils (Lichtenstein and Osler, 1964; Greaves and Mongar, 1968a, b; Greaves and Burdis, 1968).

Once the mast cell is perturbed, a cascade of steps including calcium ion influx, the activation of a serine esterase, an energy requiring phase, a second calcium dependent event and a fall in intracellular cyclic-3',5'-adenosine monophosphate (cAMP) occurs (reviewed by Austen, 1974). This cellular activation results in the release of preformed, primary mediators such as histamine and ECF-A in a fully active form and the generation and release of unstored factors such as SRS-A and PAF.

The degranulation process has been studied in the electron microscope (Uvnas, 1974; Chan, 1972). Following antigen challenge of sensitized cells the appearance of the granules changes and adjacent granules fuse to form vacuoles which eventually join with the plasma membrane. This creates open channels between the granules and extracellular space which permits the release of partially dissolved granules. Discharged granules reside in the tissue until they are removed by phagocytes.

The main constituent of the mast cell granule is a complex between basic polypeptides and the acidic polymer heparin. This complex is thought to be responsible for the cationic binding properties of the granules (Uvnas, 1974). The release of certain primary mediators, including histamine and 5'-hydroxytryptamine, has been explained as a simple cationic exchange between granule amines and extracellular cations, mainly sodium ions. While acetyl-β-D-glucosaminadase exhibits partial releasibility from the discharged or isolated granules and heparin and chymase are not released from mast

cell granules until it undergoes dissolution in high salt concentrations (Yurt et al, 1977).

The secondary mediators are a growing group of substances which are derived from the action of primary mediators upon other cell types in the immediate environment (Austen and Orange, 1975).

It is now well established in studies with human lung fragments and peripheral blood leucocytes enriched with basophils that mediator release is controlled by the levels of cAMP. Agents capable of stimulating adenyl cyclase, such as β-adrenergic catecholamines (Orange et al, 1971), histamine (Grant et al, 1974) and E series prostaglandins (Tauber et al, 1973) increase the concentration of cAMP and inhibit mediator release. Inhibitors of phosphodiesterase, the enzyme responsible for cAMP breakdown to AMP, such as methylxanthines, also block mediator release (Bourne et al, 1971b). Conversely, imidazole stimulates phosphodiesterase lowering the cellular concentration of cAMP and enhances the release of both histamine and SRS-A (Kaliner and Austen, 1974). The release of chemical mediators is also enhanced by α-adrenergic agonists and low concentrations of prostaglandin $F_{2\alpha}$ which decrease cAMP levels (Orange et al, 1971; Kaliner et al, 1972). Thus there appears to be an inverse relationship between cAMP levels and the degree of mediator release. The presence of α- and β-adrenergic receptors and receptors for histamine and prostaglandins on the target cells is also implied by these observations.

Cholinergic stimulation of sensitized lung fragments with acetylcholine or carbamylcholine chloride results in

enhancement of the IgE-dependent release of chemical mediators. The enhancement is not associated with a decrease in cAMP concentrations (Kaliner et al, 1972) and is blocked by the addition of atropine, indicating that the cholinergic receptors on the target cells in human lung tissue are of the muscarinic type. Cholinergic stimulation results in an increased tissue concentration of cyclic-3',5'-guanosine monophosphate (cGMP) (Lee et al, 1971), and the addition of 8-bromo-cyclic GMP to human lung fragments effects a dose-dependent enhancement of histamine release and SRS-A generation (Kaliner et al, 1972).

The opposing effects of cAMP and cGMP, the so-called "Yin Yang" effect (Goldberg et al, 1973), have also been observed in other tissues and cells (Turner et al, 1972; George et al, 1970). However, it is important to emphasize that there is no evidence that altering cAMP or cGMP levels can initiate secretion of mediators from mast cell or basophils per se.

Histamine can be released from rat mast cells (Kaliner and Austen, 1974) and human basophils (Ishizaka et al, 1971) by antibody directed against IgE immunoglobulin. The cross-linking of cell-bound IgE molecules by antibody triggers mediator release in a similar noncytotoxic manner as antigen. An identical release reaction has been reported by Siraganian and Siraganian (1975) using concanavalin A to induce secretion from human leucocytes. This lectin is thought to act by cross-linking the IgE molecules on the surface of the basophil since its action is inhibited by Fab anti-IgE specific for the Fc portion of the immunoglobulin molecule

(Magro, 1974). Keller (1973) observed that concanavalin A induced release of histamine from parasite infected rats but not unsensitized animals. However, Bach and Brashler (1975) suggest that these interactions may be complex in the sense that histamine was released by some lectins but not others.

All the above mechanisms are noncytotoxic processes. Anti-mast cell antibody in the presence of the second and sixth components of complement induces histamine release (Valentine et al, 1967). This reaction was shown to be cytotoxic but it has also been demonstrated that histamine was released to a greater extent than were cytoplasmic constituents.

4.2 Other stimulants of mediator release

Activated serum has been shown to initiate the release of histamine from perfused guinea pig lungs (Roch e Silva et al, 1951). Cochrane and Müller-Eberhard (1968) demonstrated that activated C3 had anaphylatoxin activity and induced the non-cytotoxic release of histamine from isolated rat peritoneal mast cells. Further studies revealed that the activity was associated with a low molecular weight cleavage fragment of C3, C3a, and that C5a was also capable of initiating histamine release (Johnson et al, 1975).

A number of relatively low molecular weight polypeptides from the venom of several insects and reptiles have the capacity to cause the release of amines from mast cells (reviewed by Habermann, 1972). A cationic protein from cobra venom, cobra venom activator (CVA), which initiates histamine release from rat peritoneal mast cells, has been described (Morrison et al, 1975a).

Polymyxin B (Norton and DeBeer, 1955) and compound 48/80 (Uvnas and Thon, 1961) are able to initiate the noncytotoxic release of histamine from rat mast cells.

Studies by Morrison et al (1975b) have provided evidence that polymyxin B and compound 48/80 induce the release of amines from mast cells by a common biochemical pathway that is distinct from the mechanism utilized by CVA protein and C3 fragments.

Several antibiotics have been described which have the capacity to transport mono and divalent cations across membranes (Caswell and Pressman, 1972). A23187 has been used extensively to examine the role of Ca^{2+} ions in the release of amines from mast cells. Foreman et al (1973) were the first to establish that this ionophore had the capacity to induce degranulation and release histamine from rat peritoneal mast cells. Recently human basophils from normal and allergic individuals have also been shown to be stimulated by A23187 to release mediators (Lichtenstein, 1975). The mechanism of ionophore mediated release appears to share some, but not all, of the pathways of other noncytotoxic mast cell/basophil activation. The available evidence suggests that the antibiotic causes an influx of Ca^{2+} ions which is a critical event in the triggering of the secretion reaction. This step appears to occur after the modulation by cAMP and before that requiring metabolic energy (Garland and Mongar, 1976).

Several other stimuli have been reported which induce histamine release from mast cells and/or basophils. These include low molecular weight cationic polypeptides extracted from the granules of various leucocytes (Kelly and White, 1973;

Ranadive and Cochrane, 1971), dextran (Garland and Mongar, 1976), adenosine-5'-triphosphate (Diamant and Krüger, 1967) and various enzymes (Uvnas and Antonsson, 1963).

5.0 PHARMACOLOGICAL MEDIATORS OF HYPERSENSITIVITY

The association of numerous mediators of immediate-type hypersensitivity with mast cells and basophils has been inferred from data provided by *in vivo* and *in vitro* models in several mammalian species. The interaction of specific antigen with IgE on the surface of mast cells and basophils results in the release of preformed basic amines and acidic peptides and the generation and release of at least three newly formed substances; slow reacting substance of anaphylaxis (SRS-A), platelet activating factor (PAF) and lipid chemotactic factors. Secondary to this, other substances such as prostaglandins and bradykinin are produced by the action of the primary mediators upon other cell types.

5.1 Histamine

The histamine present in most tissues is formed from L-histidine by a specific L-histidine decarboxylase which requires pyridoxal-5-phosphate as coenzyme. Following synthesis the histamine is stored in cytoplasmic granules within mast cells and basophils and is normally only released slowly from tissues, the half-life being about 50 days (Schayer, 1952).

Histidine decarboxylase has a pH optimum at 6-7 depending on the substrate concentration; it shifts towards the alkaline side as the concentration of substrate decreases and is accompanied by a decrease in enzyme activity (Håkanson, 1967a). The affinity of the enzyme for its substrate also varies with pH. At neutrality the affinity for histidine is higher than in more acid pH (Håkanson, 1963b). It is

also probable that the enzyme attacks the anionic form of histidine for which the percentage concentration is inversely related to K_M (Håkanson, 1967b). Therefore, at low pH the enzyme is more active but there is less histidine available and the concentration of the anionic form of histidine is a limiting factor for histamine production. At more alkaline pH the amount of available substrate is higher but the enzyme activity is lower; thus the enzyme activity becomes the limiting factor. It is, therefore, possible that pH variations, depending on substrate concentration and the effect of pH on K_M, might be one of the mechanisms of autoregulation for histamine synthesis.

The enzyme may also be controlled through its coenzyme pyridoxal 5'-phosphate which under certain conditions may react irreversibly with amines and amino acids. In such reactions Schiff's bases are transformed into cyclic compounds depending on the reacting amino acids. Pyridoxal 5'-phosphate reacts with 1-amino-2-imidazole compounds, such as histidine and histamine, to form imidazoletetrahydropyridines (Heyl et al, 1952) and with 3-hydroxyphenylaline cyclic compounds of the tetraisoquinoline type are formed (Schott and Clark, 1952). It also reacts with cysteine (Buell and Hansen, 1960) to form a thiazolidine derivative, a reaction which does not proceed via Schiff's base formation.

The formation of these compounds leads to the inactivation of the coenzyme and may be of importance in the process of inhibition of enzymatic reactions involving pyridoxal 5'-phosphate. Histidine, histamine, other amines and amino acids may, in certain conditions, form these compounds and inhibit histidine decarboxylase.

Nearly all tissues have the capacity to catabolise histamine (Schayer and Reilly, 1974). In man, histamine is largely methylated to form 1-methyl-4-(β-aminoethyl)-imidazole (methylhistamine) which is then deaminated by monoamine oxidase to form 1-methylimidazole-4-acetic-acid. A second route involves the oxidation of histamine by diamine oxidase to imidazole-4,5-acetic acid which is then conjugated and excreted as 1-riboxyl-imidazole-4-acetic acid (Schayer, 1959).

The studies of Ash and Schild (1966) and Black et al (1972) have demonstrated the presence of at least two classes of histamine receptor. The receptors involved in histamine-induced contraction of the smooth muscle of various organs including the gut and bronchus are H1-dependent. Histamine also stimulates gastric acid secretion, increases heart rate and relaxes the rat uterus; these effects are sensitive to H2-receptor antagonists.

Circulating leucocytes including basophils, neutrophils and at least certain thymus-derived lymphocytes also appear to possess H2 receptors and indeed histamine has been shown to inhibit IgE-mediated histamine release, lysozomal enzyme release and allogeneic cell killing by these respective cell types (Bourne et al, 1971; Zurier et al, 1974; Plaut et al, 1973).

5.2 Preformed chemotactic factors

Eosinophil chemotactic factor of anaphylaxis (ECF-A) was first described by Kay et al (1971) and shown to attract eosinophils selectively in modified Boyden chambers.

This preformed mediator from human lung is composed of at least two tetrapeptides, alanine-glycine-serine-glutamic

acid and valine-glycine-serine-glutamic acid (Goetzl and Austen, 1975). The chemotaxis to these tetrapeptides is thought to involve the binding of a hydrophobic domain of an eosinophil receptor with the N-terminal and activation of an ionic domain with the glutamic acid C-terminal (Goetzl and Austen, 1976).

In addition to the low molecular weight ECF-A, two other groups of preformed chemotactic factors have been extracted and/or immunologically released from human lung fragments and rat peritoneal mast cells. Eosinophil chemotactic oligopeptide with a molecular weight of 1200-2500 (Austen et al, 1976) also preferentially attracts eosinophils in vitro. A neutrophil chemotactic factor (NCF) has also been described in human leukaemic basophils (Lewis et al, 1975) and human lung fragments (Austen et al, 1976).

5.3 Slow reacting substance of anaphylaxis

The chemical structure of slow reacting substance of anaphylaxis (SRS-A) is not known (Orange and Austen, 1969) although it is thought to be an acidic sulphur-containing lipid of molecular weight 300-400 daltons (Orange et al, 1974). Its activity is measured by bioassay on an atropinized antihistamine treated guinea pig ileum (Brocklehurst, 1962). SRS-A is generated and released by antibody-mediated immunological reactions and by calcium ionophore. A small amount of SRS-A is present in a preformed state (Turnbull et al, 1976). Although other mediators may be released concomitantly evidence suggests that the release of preformed mediators and the pathway leading to the SRS-A release involves both common and different steps (Austen and Orange, 1975).

The studies of Schild et al (1951) and Brocklehurst (1962) clearly implicate SRS-A as a potentially important mediator of human allergic bronchoconstriction. SRS-A also increases vascular permeability upon intracutaneous injection (Orange et al, 1969) and decreases pulmonary compliance independent of a cholinergic mechanism when injected intravenously in the guinea pig (Drazen and Austen, 1975).

5.4 Platelet activating factor

Platelet activating factors (PAF) are a family of substances released by immunological activation of basophils and mast cells from a variety of tissues (Austen and Orange, 1975). They release 5'-hydroxytryptamine from, and in some cases aggregate, homologous platelets and are thought to be lipid-like materials (Kater et al, 1976).

5.5 Lipid chemotactic factors

Lipid factors which chemotactically attract neutrophils are released from sensitized human lung fragments and rat peritoneal mast cells following antigen challenge (Valone and Goetzl, 1978). These factors are chromatographically distinct from SRS-A and PAF and could be derived from arachidonate metabolism.

5.6 Other potential mediators

Macromolecular heparin, having a molecular weight of 750,000, has recently been shown to be released from rat mast cells by an IgE-dependent mechanism (Yurt et al, 1977) as has arylsulphatase A (Orange and Moore, 1976).

Antigen challenge of IgE sensitized human lung fragments

and peripheral leucocytes (Newball et al, 1975b) results in the release of a chymase capable of hydrolysing synthetic substrates such as N-benzyl-L-tyrosine ethyl ester. Supernatants from antigen challenged human leucocytes also appear to contain a kallikrein activity which cleaves bradykinin from its natural substrate, kininogen (Newball et al, 1975a). Whether these two activities are the same or different enzymes remains to be established.

In addition, antigen challenge of sensitized human (Piper and Walker, 1973) and guinea pig (Dawson et al, 1976) lung results in the release of various prostaglandins and thromboxanes.

SECTION II - THE BASOPHIL LEUCOCYTE

1.0 HISTORICAL BACKGROUND

The basophil leucocyte was first observed in the blood of patients with chronic myeloid leukaemia by Paul Ehrlich (1891). He recognized them as a form of polymorphonuclear granulocyte derived from the bone marrow, distinct from tissue mast cells which he had previously described and differentiated from the plasma cells of Waldeyer (1875). The major problem in the study of blood basophils is their relative paucity and lack of obvious function. Prior to 1953, when the first direct chamber counting method was developed by Moore and James (1953), very little information on the chemistry and pharmacology of these cells was available. However, when reviewed by Michels (1938) their morphology and distribution in many organisms had been studied in depth, along with their variation under numerous pathological conditions.

Interest in basophils has been stimulated in the last decade by findings that implicate them in both immediate and delayed types of hypersensitivity. Ishizaka and Ishizaka (1971) demonstrated that basophils specifically bind homocytotropic antibody, thus sensitizing them for antigen induced mediator release. It is possible, therefore, that basophils as well as mast cells participate in atopic allergic disease. The application of modern histological methods for fixing and processing tissue has implicated basophils in several forms of delayed type hypersensitivity (Dvorak *et al*, 1971).

2.0 ORIGIN AND DIFFERENTIATION

The basophil is a polymorphonuclear granulocyte which differentiates in the bone marrow. The mature form circulates in the blood of most vertebrates including man (Michels, 1938) where its life span is 8-12 days.

Basophil differentiation has not been studied in man but several studies have been carried out on the guinea pig (Winqvist, 1963; Terry et al, 1969; Dvorak et al, 1972). Early myelocytes are the first cell in which basophil-specific granules appear. These cells have a prominent Golgi apparatus and abundant rough endoplasmic reticulum.

Basophil granules arise as small vesicles which pinch off from the Golgi cisternae. These granules subsequently aggregate to form larger, but still immature, structures lying in the peripheral cytoplasm. They show reticular, mottled or loose lamellar ultrastructure and have only a thin hole around the matrix in contrast to the broad rim surrounding the majority of the definitive granules. With further development the granule precursors organise to form the highly ordered patterns characteristic of mature granules. The origin of basophil granules from the Golgi apparatus is, therefore, comparable to that of the secretory granules of neutrophils, eosinophils and other cells including mast cells.

3.0 DISTRIBUTION AND FREQUENCY

The distribution and frequency of basophils varies in certain physiological and pathological conditions.

3.1 Normal

Basophils, which account for less than 1% of the circulating leucocytes and about 0.3% of the nucleated cells of the bone marrow, are the least numerous human granulocyte (Juhlin, 1963a). Slight variations in basophil frequency have been reported with age and sex. Their percentage is somewhat lower in children (Mitchell, 1958) while young adult females have the highest basophil counts (Thonnard-Neumann, 1963).

With few exceptions a 0.5-1.0% level is maintained in most mammalian species. However, in rabbits and camels basophils have been reported to account for up to 10% of the circulating leucocytes. In lower vertebrates, as a rule, basophils comprise a much larger percentage, 7-33%, while in most fish they are extremely rare (Michels, 1938).

3.2 Variations

Basophil counts are altered by a variety of hormones. Cortisone and adrenocorticotrophic hormone induce a fall in the number of circulating basophils in man, but to a lesser extent than eosinophils (Keleman and Bikich, 1956). Boseila *et al* (1959) have suggested that the numbers of basophils may be controlled by the action of circulating thyrotrophin and thyroid hormone. L-thyroxin depresses basophil numbers whereas metabolically inert D-thyroxine increases basophil levels, possibly by inhibiting thyrotrophin secretion.

Sex hormones influence the number of circulating basophils which also vary during the menstrual cycle (Boseila, 1959). Their numbers are increased under the influence of oestrogens and decreased by luteal or progesterone action. In the rabbit a fall in the number of circulating basophils occurs at the time of ovulation; simultaneously an accumulation of basophils takes place in ovaries and oviduct vessels and around the recently ruptured follicles (Zachariae et al, 1958).

Physiological variations in the number of basophils in man were studied by Rorsman (1962) who noted that food intake, moderate exercise and mechanical lesion of the small blood vessels did not influence the levels of circulating basophils.

Variations in basophil numbers have been reported in a variety of diseases. They can be dramatically increased in myeloproliferative disorders, particularly chronic granulocytic leukaemia where they may number more than $90 \times 10^{6}.ml^{-1}$ (Youman et al, 1973) and can account for over 90% of the circulating leucocytes (Austen et al, 1974). Elevation in the blood basophil count has also been reported in ulcerative colitis, myxoedema, chronic sinusitis, small-pox, chicken pox, polyerythaemic vera and in various atopic states (Fredricks and Maloney, 1959; Juhlin, 1963b; Shelley and Parnes, 1965). Peripheral basophil numbers are low in urticaria, acute infection, following x-radiation or chemotherapy and immediately following anaphylaxis (Rorsman, 1962; Shelley and Juhlin, 1961).

In certain experimental animals the number of basophils can be greatly increased by the administration of various antigens. In the guinea pig substantial elevation of bone marrow and circulating basophils can be achieved by repeated injection of large quantities of heterologous serum or blood (Ringoen, 1923; Chan and Yoffey, 1960; Winqvist, 1960). This phenomenon has been used by Dvorak et al (1974) to prepare large numbers of purified basophils. The mechanism responsible for the increased basophilopoiesis has not been established but the basophil response is highly selective in that there is little change in the overall blood leucocyte or bone marrow nucleated cell count. The number of eosinophils may also increase but the increment is smaller and slower to develop (Albanus and Winqvist, 1961).

In 1923 Ringoen described a pronounced basophil granulocytic exudation in the subcutaneous connective tissues of guinea pigs 4-8 days after the injection of egg white. Since then basophil leucocytes have been reported to infiltrate certain tissues in various diseases, without the presence of a peripheral basophilia.

Basophils are characteristically present in reactions of delayed type hypersensitivity, including allergic contact dermatitis, skin allograft and tumour rejection and hypersensitivity to protein antigens (Dvorak and Mihm, 1972; Dvorak, 1971; Wolf-Jürgensen, 1966). Their presence has also been reported in other diseases of the skin where a component of cell-mediated hypersensitivity may exist, including eczema, psoriasis and drug reactions, dermatitis herpetiformis, insect bites and photo-contact dermatitis

(Juhlin, 1963c; Aspegren et al, 1963b; Fregert and Rorsman, 1964). Felarca and Lowell (1971) described basophil infiltration into allergen-specific skin window sites in some patients with atopic disease.

Basophils have also been reported in other tissues. Colvin et al (1974) observed their presence in drug-related allergen interstitial nephritis. They are also a component of the inflammatory infiltrate in renal allografts undergoing active cellular rejection (Colvin and Dvorak, 1974).

4.0 MORPHOLOGY

Basophils from various species have been studied in the electron microscope and substantial differences in their ultrastructural appearance have been described. Again, such studies on normal human basophils have been hampered by their paucity in peripheral blood and susceptibility to damage. However, the use of animal models, especially the guinea pig, has provided a great deal of information on their nuclear and cytoplasmic structure.

In vital films studied by phase-contrast microscopy, human basophils measure 10-14 μm in diameter and are the smallest of the granulocytes (Ackerman and Bellios, 1955).

4.1 Nucleus

Basophils have a band or bilobed nucleus with dense irregular condensations of chromatin along a thin nuclear membrane. The nucleus is quite similar to that of the neutrophil and eosinophil although the segmentation of the nuclear lobes is less pronounced.

4.2 Granules

The characteristic cytoplasmic granules of the human basophil are electron dense, round, oval and angular shaped, membrane-bound structures that measure up to 1.2 μm in their largest dimensions (Zucker-Franklin, 1967). The sub-structure of basophil granules is composed of dense particles embedded in a less dense matrix. Those particles, present within a single granule, can vary in size and are generally rounded but have irregular peripheral borders. Some granules contain complex collections of membranes. A second set of smaller granules is generally found between the lobes of the

nucleus (Hastie, 1974). These are bound by thinner membranes and contain uniform, lightly dense material.

The granules of the guinea pig basophil on average measure slightly more than 0.5 by 1.0 μm but occasionally even 1 by 2 μm. According to the plane of the section they appear oval or spherical and may show a repeating 130 Å banded pattern, a hexagonal array or a rectangular lattice (Terry et al, 1969; Winqvist, 1963). Some granules contain only uniform, finely granular material with no discernible repeating pattern. Whatever the substructure, the granules are frequently surrounded by a hole of varying breadth. This rim is not entirely empty but contains material of low electron density that sometimes forms concentric or radical filaments and separates the granule matrix from the membrane.

Rabbit basophil granules are composed of very dense particles which may be arranged in a regular array (Wetzel, 1967).

4.3 Other organelles

The cytoplasm of the guinea pig basophil contains small vesicles and vacuoles, some of which are in continuity with cytoplasmic granules. Relatively few mitochondria and ribosomes are present. The former are long and slender, about 0.3 by 1.5 μm, with numerous transverse cristae. The Golgi apparatus, usually situated in the central part of the cell, and an endoplasmic reticulum are present in mature cells but these, along with ribosomes, are more abundant in immature elements. One or two centrioles have been observed between the nucleus and the Golgi apparatus (Winqvist, 1963). Microfilaments and microtubules have

been identified. The plasma membrane is thin, about 11 nm, and frequently shows short surface villi and large blunt pseudopodal processes.

Similar organelles have been described in other species, including man. In all species studied the mature cells contain large numbers of glycogen particles which are often closely associated with the cytoplasmic granules and vesicles.

5.0 BIOCHEMISTRY AND PHARMACOLOGY

The elusiveness of the basophil has, until recently, complicated the analysis of its biochemical constituents. In fact, much of the available data has been derived from studies using extremely impure cell populations and will require further confirmation. However, cytochemical evidence, when available, is much more reliable.

5.1 Pharmacological mediators

Several low molecular weight substances are released from leukaemic basophils following an anaphylactic reaction. These include at least four substances, histamine, SRS-A, ECF-A and PAF (Lewis *et al*, 1975) which are also found in mast cells.

5.1.1 Histamine

Since Dale (1913) first described histamine as a mediator of immediate-type hypersensitivity there has been increasing evidence that the basophil is the site of storage of this amine in blood. Histamine content was found to correlate with basophil frequency in both normal volunteers and patients with chronic myelogenous leukaemia (Ehrich, 1953; Valentine *et al*, 1955). Graham *et al* (1955) originally estimated that half of the blood histamine was confined to basophils. The release of histamine from human basophils was conclusively demonstrated by Ishizaka and co-worker (1972) and it is now generally accepted that virtually all the histamine present in human and guinea pig blood is contained within basophils. In contrast, rabbit basophils account for only one-quarter of the blood histamine, the

remainder being associated with the platelets (Code, 1952).

Assuming that all the histamine is present within basophils it can be estimated that in man each cell contains 1-2 pg (Graham et al, 1955). Cell disruption studies have revealed that the majority of the histamine present within human basophils is located within the cytoplasmic granules (Pruzansky and Patterson, 1967).

Basophils are capable of synthesizing histamine from L-histidine, the reaction being catalysed by a histidine decarboxylase which has been detected in blood (Hartman et al, 1961; Ackerman, 1963). As the rate of histamine formation was proportional to the number of basophils (Lindell et al, 1961), the enzyme is presumably in these cells. Indeed, guinea pig basophils have been reported to take up very little extracellular histamine (Galli et al, 1976) although Day et al (1974) have claimed that human basophils do incorporate this amine and store it in their granules. Galli et al (1976) have also provided evidence that circulating guinea pig basophils are capable of histamine synthesis.

5.1.2 Other mediators

Both normal and leukaemic human basophils have been shown to release SRS-A (a mediator which is largely pre-formed, the majority being generated in the course of immediate hypersensitivity reactions (Grant and Lichtenstein, 1974; Clark et al, 1976)) and ECF-A (Parish, 1972).

Rat leukaemic basophils, like rat peritoneal mast cells, may contain 5-hydroxytryptamine, but human basophils apparently lack this substance (Humphrey and Jaques, 1954).

PAF has been identified in rabbit (Siraganian and Osler, 1971) and human (Benveniste, 1974) leucocytes. That the source of this chemical mediator is the basophil is indicated by the fact that PAF can be released from suspensions of human leukaemic basophils upon interaction with calcium ionophore (Lewis et al, 1975).

Several other possible pharmacological mediators have been reported to be present in human basophils or to be released from them by antigen challenge. These include a kallikrein activity (Newball et al, 1975a) and a chemotactic factor selective for neutrophils (Lewis et al, 1975).

5.2 Acid mucopolysaccharides

Basophils from all mammalian species studied exhibit metachromatic colouration of their granules following staining with certain basic dyes, such as toluidine blue, thionine and methylene blue (Ackerman, 1963). On this basis, basophil granules have been thought to contain sulphated acid mucopolysaccharides or proteoglycans and, by analogy with mast cells, this was presumed to be heparin (Lagunoff et al, 1964).

Several workers have reported the presence of chondroitin sulphate in rabbit and human basophils (Olsson, 1971; Olsson et al, 1968). Recently Orenstein and co-worker (1978) have characterized the acid mucopolysaccharide present in guinea pig basophils in vivo and in vitro, using ^{35}S labelling. They found that the majority of these macromolecules was a mixture of chondroitin sulphate, dermatan sulphate and small amounts of heparin sulphate. No heparin was detected.

5.3 Enzymes and other constituents

The other cellular constituents have been poorly characterized. As measured cytochemically, basophils are said to lack hydrolytic enzymes such as alkaline phosphatase, aminopeptidase and non-specific esterases (Ackerman, 1963). Recently, Dvorak *et al* (1977) using biochemical and ultrastructural techniques demonstrated proteolytic enzyme activity in purified guinea pig basophil granules. These preparations contained a mixture of neutral esterases: proteases including caseinolytic activity and both trypsin- and chymotrypsin-like serine hydrolyases were identified. Peroxidase activity has been reported in human basophil granules but it is normally lacking in basophils from the rabbit and guinea pig (Ackerman, 1963; Ackerman and Clark, 1971). Guinea pig basophils have plasminogen activator activity associated with their plasma membrane (Dvorak *et al*, 1978).

A variety of oxidative enzymes, including diaphorases and several dehydrogenases are present in human basophils (Balogh and Cohen, 1961; Ackerman, 1963). Human leukaemic basophils are capable of generating and releasing superoxide radicals after immunological or non-immunological stimulation and also contain superoxide dismutase (Henderson and Kaliner, 1978).

Glycogen particles are abundant in the cytoplasm, often in close approximation to and sometimes within the granules, of all species studied (Dvorak and Dvorak, 1975).

6.0 STORAGE AND RELEASE OF PHARMACOLOGICAL MEDIATORS

The cytoplasmic granules of the basophil hold large stores of potentially important pharmacological agents. Under a variety of conditions, including hypersensitivity reactions, these mediators are released and others formed. The processes and reactions involved have been described in Section I (part 4) and are essentially the same as for mast cells.

7.0 PHAGOCYTOSIS AND PINOCYTOSIS

Basophils have been reported to ingest sensitized erythrocytes and antigen-antibody complexes but not colloidal carbon (Sampson and Archer, 1967; Dvorak et al, 1972). However, their phagocytic ability is appreciably less than that of neutrophils and eosinophils and it does not seem to be one of their major functions.

In contrast, basophils have a substantial capacity for pinocytosing certain solutes. Neutral red, toluidine blue and thionine are all ingested by basophils (Sabin, 1923; Chan and Yoffey, 1960). In an electron microscopic study Dvorak et al (1972) demonstrated that horseradish peroxidase is transported to the cytoplasmic granules by a pinocytotic process.

8.0 CHEMOTAXIS

Human and guinea pig basophils have been shown to be responsive chemotactically to "lymphokines" elaborated from sensitized lymphocytes cultured with specific antigen and products of complement activation (Boetcher and Leonard, 1973; Ward et al, 1975). In addition, diffusates from lung fragments challenged with allergen, the plasma enzyme kallikrein and an additional complement component, $\overline{C567}$, have been found to be weakly chemotactic for human leukaemic basophils (Kay and Austen, 1972). Guinea pig basophils are also attracted by products present in bacterial culture supernatants (Ward et al, 1975).

The chemotactic response of basophils is not specific since other cell types respond to the same agents. However, the kinetics of the basophil migration is distinct, they respond rapidly and reach a plateau as early as 1 hr whereas neutrophils and macrophages do not achieve such levels for 2 and 3-5 hr respectively.

9.0 CUTANEOUS BASOPHIL HYPERSENSITIVITY

Several forms of delayed onset, lymphocyte mediated hypersensitivity are characterized by an extensive infiltration of basophil leucocytes (Dvorak and Dvorak, 1974). Such reactions have been termed cutaneous basophil hypersensitivity or CBH. These were first observed in guinea pigs with so-called "Jones-Mote" hypersensitivity, a form of cell mediated reactivity to protein antigens following sensitization without the use of mycobacterial adjuvants. In contrast to classic, tuberculin-type delayed hypersensitivity CBH skin reactions are relatively non-indurated, are characterized by extensive infiltration of basophils (one-third to one-half of total infiltrates), contain substantial amounts of histamine, are affected by lymphocytes which are difficult to tolerize and lack extensive fibrin deposits (Dvorak and Hammond, 1978).

CBH reactions may be elicited by skin testing as early as 4 or 5 days after sensitization and are maximal by 6 to 7 days (Richerson *et al*, 1970); after two or more weeks CBH reactivity often wanes and may be superseded by the appearance of "late reactions", complex lesions comprised of sequential but overlapping components of cutaneous anaphylaxis, Arthus reactivity and a residual basophil component (Colvin *et al*, 1973).

While the expression of CBH has been most carefully studied in the skin of guinea pigs, cell mediated reactions rich in basophils also occur in other organs and species (Dvorak and Dvorak, 1974). Examples include peritoneal rejection of ascites tumour cells in guinea pigs,

experimental allergic encephalomyelitis in rats and immunity to parasites (Ogilvie, 1974). Significant species differences exist in the frequency with which basophils participate and basophil infiltration is generally greater in guinea pigs than in other species studied.

Sensitized lymphocytes are essential to the induction and expression of these reactions (Dvorak and Dvorak, 1974) and it is likely that basophils are attracted to local skin test sites by lymphocyte products. Askenase (1973) has claimed passive transfer of CBH with immune serum although other attempts to implicate antibody in the pathogenesis of these reactions have been unsuccessful.

Basophils have also been described in the cellular infiltrates in various forms of delayed skin reaction by the use of skin window techniques (Aspegren et al, 1963a). Recently they have been identified in human tissue sections from patients with allergic contact dermatitis and classical delayed hypersensitivity reactions to microbial antigens (Dvorak and Mihm, 1972).

Degranulation of the type seen in anaphylaxis has only rarely been observed in basophils infiltrating CBH lesions in the guinea pig. However, basophils participating in cell mediated reactions in man and certain forms of CBH in guinea pigs (Dvorak et al, 1973) regularly exhibit striking alterations in their cytoplasmic granules which develop progressively over a period of days as the reaction evolves.

10.0 BASOPHILS AND MAST CELLS

In various species there is a reciprocal relationship between the number of mast cells and basophils (Michels, 1938). Thus rats, mice and many types of fish, in which basophils are extremely rare, have numerous fixed tissue mast cells whereas the opposite is true of the rabbit. This relationship has supported the widely held view that the two cell types complement each other and have similar or even identical functions. In some lower vertebrates it is impossible to distinguish basophils from mast cells. Differences occur among mast cells of different species although this does not seem so marked for basophils.

There is, however, a danger of drawing too close an analogy between the two cell types. Basophilic leukaemia and systemic mastocytosis are discrete afflictions involving the two cell types independently of each other. Therefore, different mechanisms must be involved in controlling their numbers and the cells have been shown to arise from different precursors. Nevertheless, they have a number of structural features and activities in common.

Basophils and mast cells are the only cell types with receptors for homocytotropic antibody through which the release of histamine and other pharmacological mediators can be activated by antigen and other stimuli. Both cell types can decarboxylate L-histidine and bind the resultant histamine in their granules which also contain zinc (Smith et al, 1969). The granules are metachromatic but that of the basophil is more labile, a property that can be used to differentiate the two cell types (Padawer, 1959). In

man, basophil granules are water soluble whereas mast cell granules are not. Mast cells contain heparin; however, the acid mucopolysaccharide of basophils has been shown to be a mixture of chondroitin, dermatan and heparin sulphate. Recently Galli et al (1978) developed a specific anti-basophil serum which caused histamine release from basophils but also reacted with mast cells. Therefore, there are antigenically similar structures on the surface of both cell types.

Apart from its motility, the chief morphological features which distinguish the basophil from the mast cell are its smaller size, more rounded shape, its relatively scanty cytoplasm and usually obscured segmented nucleus. Ultrastructurally human basophil granules display either homologous or coarsely stripped appearance in the electron microscope. In contrast, mast cell granules show complex and abundant whorls. Within any one species the morphology of mast cells differs from that of the basophil, but the basophil of one species can resemble the mast cell of another, and vice versa.

11.0 POSSIBLE ROLE OF THE BASOPHIL LEUCOCYTE

The function(s) of basophils are poorly understood but they are probably related to the release of mediators contained within their cytoplasmic granules. The physiological role of the mast cells is also unknown although it is presumably not present only to undergo hypersensitivity reactions. Asboe-Hansen (1973) has postulated that mast cells participate in all diseases of connective tissue and that no new formation of such tissues takes place without a demonstrable activity of mast cells. Mast cells have also been associated with the host defence against parasites (Ogilvie and Jones, 1971) where IgE antibody is also involved.

Norman (1975) has suggested that mast cell degranulation serves to facilitate an influx of high molecular weight IgG and IgM from the vasculature for complexing with antigens extravascularly. This theme has been developed by Padawer (1978) to incorporate the basophil. He speculates that the basophil coated with IgE, moves into tissues in response to chemotactic attraction, not as Norman suggests to transfer IgE to the mast cell but to release its mediators upon interaction with extracellular antigen. This would cause oedema accompanied by influx of IgG, IgM and unbound IgE. As events proceed, the unbound IgE could interact with mast cells triggering a second response, leading to the further involvement of antibody and the attraction of various inflammatory cells.

This is partly supported by the work of Dvorak and Dvorak (1974) on CBH. The release of vasoactive amines

may be responsible for certain features of these reactions including vasodilation and hence erythema and increased vascular permeability leading to compaction, oedema and leakage of fibrinogen and other clotting factors. By releasing ECF-A, basophils could also be responsible for attracting eosinophils which are regularly present in these reactions.

It is still possible that the mast cell and basophil are complementary. The long-lived sessile mast cells on site ready to respond instantaneously and the basophil able to migrate to areas of need. Mast cells in all tissues being virtually responsible for repair after injury or infection, with basophils playing their part when required. During evolution as organisms developed parasites, IgE was developed as a possible answer to the problem. In the presence of an infection, parasite specific IgE bound to mast cells or basophils could be activated, mediators released and the parasite expelled. The fact that mast cells and basophils are able to take part in anaphylactic reactions is a failure to limit their proper response which leads to disease.

CHAPTER II - AIMS OF THE PRESENT STUDY

There is increasing evidence suggesting a regulatory role for the eosinophil leucocyte in immediate type (type I or anaphylactic) hypersensitivity reactions. They may be involved at all stages of the allergic response, i.e. mediator release, mediator inactivation and mediator replenishment.

Eosinophils have been shown to accumulate at the site of allergic reactions following antigen challenge (Kay, 1970a; Samter et al, 1953; Fowler and Lowell, 1966). This accumulation is directed by the release of ECF-A from mast cells (Kay and Austen, 1971). Recently, histamine and imidazoleacetic acid have also been shown to be selectively chemotactic for human (Clark et al, 1975; Turnbull and Kay, 1976) and guinea pig eosinophils (Jones and Kay, 1977).

Following an anaphylactic reaction an eosinophil-derived inhibitor of histamine release (EDI) may inhibit further mediator release (Hubscher, 1975a). This factor is thought to be a prostaglandin (Hubscher, 1975b).

The eosinophil's relatively high content of histaminase (Zeiger and Colten, 1977) and arylsulphatase (Wasserman et al, 1975) may have a biological role in the inactivation of histamine and SRS-A, respectively. Other antihistamine effects have been reported in a variety of experimental models. The first evidence that eosinophils could exert an antihistamine action was proposed by Kovacs (1950) when he showed that an eosinophil-rich leucocyte suspension, injected intraperitoneally, could lessen the effect of histamine-induced bronchospasm in guinea pigs. Vercauteren and Peeters (1952) and Vercauteren (1953) demonstrated that

preparations of horse eosinophil granules and arginine suppressed the effect of histamine on guinea pig ileum. They suggested that the arginine-rich proteins from the granules could have antihistamine properties. Archer (1960) noted that the weal expected after an intradermal injection of histamine was inhibited if the skin site was previously injected with a suspension of eosinophils. The same author reported that injections of horse eosinophils protected guinea pigs from an histamine aerosol (Archer et al, 1962).

Eosinophils may also influence the repair process which leads to mediator replenishment. It was shown that when guinea pigs were depleted of eosinophils, by anti-eosinophil serum, histamine reaccumulation at the site of passive cutaneous anaphylaxis was considerably accelerated (Jones and Kay, 1976); thus suggesting that eosinophils may inhibit mast cell regranulation. An alternative possibility is that they suppress the maturation or differentiation of new mast cells.

Therefore, eosinophils may provide a homeostatic function, "dampening" and "curtailing" the effects of pharmacologically active mediators by influencing their release, inactivation and replenishment.

Apart from its regulatory role in type I hypersensitivity reactions the eosinophil has been shown to affect mast cells in other ways. Histamine release is thought to involve the exposure of membrane-free granules, either by expulsion or retention in vacuoles connected to the outside, to cation-containing extracellular fluids which cause amine release by simple cation exchange (Uvnas, 1974). Eosinophils have been

seen to phagocytose mast cell granules (Welsh and Geers, 1959). Components of eosinophil granules have also been reported to cause mast cell disruption (Archer and Jackas, 1965). Archer and McGovern (1968) investigating the role of the eosinophil in parasite infections in rats observed changes in mast cell populations. As the eosinophilia developed the mast cells degranulated and after about 10 days new mast cells were forming.

These observations involve the "interaction" between eosinophils and cells of the mast cell/basophil series. To investigate these interactions a single cell system was developed to study aspects of histamine storage and synthesis. The eventual aim was to determine whether histamine metabolism was altered in a cell which had undergone an anaphylactic reaction and if eosinophils or eosinophil-derived products influenced that metabolism.

Histamine is not the only mediator released from basophils or mast cells during a type I hypersensitivity reaction. However, its synthesis is possibly the simplest to study since it is formed from a single molecule, histidine, and it is easily detected and measured. Other pharmacologically active mediators are present in preformed or precursor states. Their molecular structure is more complicated and they are more difficult to measure and quantify.

Guinea pig ECF-A is probably an acidic peptide or series of peptides similar to the human forms which have been sequenced (Goetzl and Austen, 1975). It is unlikely that the basophil or mast cell possess specialized transport

systems for all its constituent amino acids since the amino acid pool within the cell is probably capable of supplying all that is required. The structures of the other primary mediators, SRS-A and PAF, are not known. SRS-A is thought to be an acidic sulphate ester and PAF a phospholipid (Austen and Orange, 1975). Therefore, modulations of their metabolism would be difficult to measure.

Prostaglandins, secondary mediators, have been measured in sensitized guinea pig lung diffusates (Dawson et al, 1976). However, their detection is very complicated, requiring gas liquid chromatography and mass spectrometric analysis. Therefore, histamine is the logical pharmacologically active mediator to use as a measure of mediator variations within the basophil.

The histamine found within mast cells and basophils could be derived from two sources. It could be the result of direct uptake of histamine from the extracellular fluids particularly after an anaphylactic reaction when high concentrations of histamine could be present in the microenvironment surrounding the cells. Furano and Green (1964) have supported this concept by showing that isolated rat mast cells take up and store histamine. Basophils and mast cells are also capable of forming histamine by the decarboxylation of L-histidine (Schayer, 1956; Lindell et al, 1961). Therefore, the intracellular histamine could also be derived from the uptake of exogenous histidine and its decarboxylation within the cells.

The development of a method by which guinea pig basophils can be obtained in relatively high concentrations

(Dvorak *et al*, 1974) afforded the possibility of an experimental model which could be used to study mast cell/basophil function. In the present thesis this model has been developed and utilized to study histidine and histamine uptake and histamine formation from exogenously incorporated histidine.

The guinea pig is and has been a useful experimental animal to use in the study of anaphylaxis. It can be easily sensitized to a variety of antigens either actively or passively. The whole animal or some of its organs respond very strongly to an antigen challenge permitting the study of quantitatively graded allergic reactions. Perhaps more importantly the immunologically released mediators and mechanisms of release are almost identical to those encountered in man.

Therefore, the uptake of histidine and histamine and histamine synthesis *de novo* by guinea pig basophil leucocytes was used to study variations in histamine storage.

Before the effect of eosinophils on histamine replenishment after an anaphylactic reaction could be examined the experimental model had to be investigated. To this end the uptake of histidine and histamine and histamine synthesis *de novo* was examined in depth. If an insight into the control of these processes could be obtained then the influence of the eosinophil on these controlling factors may provide an explanation of the "dampening" effect of eosinophils on histamine replenishment.

CHAPTER III - MATERIALS AND METHODS

SECTION I - BUFFERS AND REAGENTS

1.0 BUFFERS

All chemicals were analytical grade obtained from BDH Chemicals Ltd., Poole, Dorset, unless otherwise stated.

1.1 Tyrode's buffer

Tyrode's buffer was composed of the salts as originally described (Tyrode, 1910). It was prepared by mixing 40 ml Tyrode's "A" (see below), 1.0 g glucose, 1.0 g $NaHCO_3$, 1.0 ml 20% $CaCl_2$ and 0.4 ml 25% $MgCl_2$ and making the volume up to 1 litre with distilled water. The pH was adjusted to 7.25-7.4.

Tyrode's "A" was composed of 200 g NaCl, 5 g KCl, and 1.62 g $NaH_2PO_4.2H_2O$ dissolved to a final volume of 1 litre with distilled water.

1.2 Phosphate buffered saline (PBS)

This was prepared by mixing 6.8 g NaCl, 1.48 g Na_2HPO_4 and 0.44 g KH_2PO_4 and adjusting the volume to 1 litre with distilled water.

1.3 PBS/HSA

PBS/HSA is a buffered salt solution composed of phosphate buffered saline supplemented with 0.3 $mg.ml^{-1}$ human serum albumin (a gift from the South-East Scotland Regional Blood Transfusion Service) and 0.1% EDTA and adjusted to pH 7.3.

1.4 Modified phosphate buffered saline

Modified PBS was prepared by dissolving 6.8 g NaCl, 1.48 g K_2HPO_4, 0.43 g KH_2PO_4 and 1 g glucose up to 1 litre with distilled water.

Choline substituted modified PBS was prepared as above but adding 16.3 g choline chloride in place of the NaCl.

1.5 Eagle's Minimal Essential Medium without histidine

This contained the constituents as described by Eagle (1959) with the exception of L-histidine. It was prepared as a ten times concentrate by dissolving the constituent amino acids in 95 ml ten times concentrated Eagle's balanced salt solution with Earle's salts and 10 ml one hundred times concentrated Minimal Essential Medium Vitamins (both from Gibco Bio-Cult Ltd., Paisley, Renfrewshire; amino acid from Sigma Chemical Co., Poole, Dorset). The L-tyrosine was dissolved in 10 ml of the balanced salt solution at pH 10 before being added to the rest of the solution. The medium was sterilized by filtration and kept at 4^{o} until required. To use, the prepared medium was diluted 1 in 10 with distilled water or distilled water with sufficient guinea pig serum to give a final concentration of 10% and 0.29 $mg.ml^{-1}$ L-glutamine added. The solution was then adjusted to pH 7.25-7.4.

2.0 STAINS

2.1 Basophil stain

A differential basophil count can be carried out in a Neubauer haemocytometer using the stain developed by Cooper and Cruickshank (1966) with the addition of guinea pig serum at a final concentration of 10%.

The stain was prepared by mixing 25 ml of 0.5% cetyl-pyridinium chloride, 25 ml of distilled water and 20 ml of 0.8% toluidine blue in 5% $(Al)_2$ $(SO_4)_3$. The solution was filtered regularly and to use 1 part cell suspension was added to 8 parts stain and 1 part guinea pig serum.

2.2 Trypan blue

1% aqueous trypan blue was added 1:1 with the cell suspension.

2.3 White cell diluting fluid

A stock was prepared by adding 0.1 g gentian violet to 100 ml of 1% acetic acid. To use, the stock was diluted 1:10 with 1% acetic acid and 1 part cell suspension added to 9 parts stain.

3.0 REAGENTS

3.1 Toluene based scintillator

The liquid scintillator used to measure the amounts of radioisotope present on the chromatograms was prepared by dissolving 4 g 2,5-diphenyloxazole (PPO) and 0.2 g 1,4-di-2-(5-phenyloxolyl)-benzene (POPOP) in 1 litre toluene (Koch Light Laboratories Ltd., Colnbrook, Bucks.).

3.2 Chromatogram stain

The positions of the authentic standard histidine and histamine on the developed chromatograms were identified by diazotised sulphanilic acid (Ames and Mitchell, 1952). The dried chromatograms were first sprayed with a mixture of 5 parts ice-cold 5% $NaNO_2$ and 1 part 0.9% sulphanilic acid in 9% HCl and then with 5% Na_2CO_3 using a laboratory spray gun (Shandon Scientific Co. Ltd., London).

3.3 Preparation of antigens

The sheep's blood used for injections was obtained every two weeks by venepuncture and collected into sterile Alsever's solution (1:1). The sheep's blood cell lysate used as the antigen for anaphylactic histamine release was prepared when required by centrifuging 10 ml of sheep's blood at 150 g for 10 min and resuspending the pelleted cells in 5 ml distilled water.

The ovalbumin used to stimulate a basophilia was a 50 $mg.ml^{-1}$ solution of ovalbumin (crude powder grade II, Sigma Chemical Co., Poole, Dorset). For anaphylactic histamine release albumin, chicken egg five times crystallized (Koch-Light Laboratories Ltd., Colnbrook, Bucks.) was used.

SECTION II - PURIFICATION OF CELLS

1.0 BASOPHIL LEUCOCYTES

Basophils from the bone marrow and peripheral blood were purified by the method of Dvorak *et al* (1974) with some minor modifications.

1.1 Bone marrow derived cells

Basophilia was induced in 250-400 g Dunkin-Hartley guinea pigs of either sex by 12 daily intraperitoneal injections of 1 ml sheep's blood or ovalbumin (see Section I, part 3.3). The day after the final injection the animals were killed by dislocation of the vertebrae, the neck opened and the blood collected in a glass universal container. The blood was allowed to clot and the serum removed for use later in the purification or stored at -70^{o} until required. After exsanguination the bone marrow was harvested from the femora, tibias and humeri by flushing the shaft with Eagle's minimal essential medium (Wellcome Reagents Ltd., Beckenham, Kent), pH 7.3-7.4. A cell suspension was prepared by gentle pipetting and fatty material and pieces of broken bone removed by filtration through a plastic sieve. A differential cell count was performed on the cell suspension obtained from each animal and those containing less than 5% basophils were discarded.

Pooled, basophil-rich bone marrow preparations, usually from two animals, were centrifuged at 100 g for 10 min at 4^{o} and resuspended in 30 ml of a mixture of 1 part Eagle's minimal essential medium and 1 part normal guinea pig serum, pH 7.25-7.4. The cells were incubated with 10 $mg.ml^{-1}$ ex-carbonyl iron particles (Koch-Light Laboratories Ltd., Colnbrook, Bucks.) in a shaking water bath at 37^{o} for 30 min.

Phagocytic cells, mainly neutrophils and monocytes, and residual iron particles were removed by passing the cell suspension six times through a piece of tubing fixed in a magnetic field.

The phagocyte-free suspension was centrifuged at 100 g for 10 min at 4°, resuspended in 8 ml PBS/HSA and layered on to a continuous Ficoll density gradient. The gradient was prepared by placing 5 ml of 43% Ficoll 400 (Pharmacia (G.B.) Ltd., London) in PBS/HSA into a 100 ml round-bottomed glass centrifuge tube and layering on to this 10 ml of decreasing concentrations (10.5-5.5%) of Ficoll in PBS/HSA in 1% steps. The tube was allowed to equilibrate overnight at 4°.

The linearity of the gradient was confirmed using a Bellington and Stanley "Abbe 60" refractometer, and comparing the refractive angle of each fraction to a standard curve of Ficoll solutions of known concentration and density.

The gradient was centrifuged at 85 g for 15 min at 4° and 4 ml fractions collected from a point immediately above the 43% Ficoll cushion using a glass capillary tube connected to a peristaltic pump. Each fraction was centrifuged at 100 g for 10 min at 4°, washed with Tyrode's buffer and resuspended in 8 ml of the same buffer. Differential cell counts were performed to identify the basophil containing fractions which were pooled and adjusted to the required concentrations.

1.2 Peripheral blood derived cells

Basophil enriched cell suspensions were purified from the blood of sheep's blood treated guinea pigs using the same technique as for the bone marrow derived cells with a few modifications to remove the red cells.

The blood was collected in plastic universals containing sufficient heparin (Weddel Pharmaceuticals Ltd., London) to provide a final concentration of 10 units.ml^{-1}. Because of the low number of leucocytes present the ex-carbonyl iron step was omitted since it resulted in the loss of a large number of cells.

The blood from 8-10 animals was pooled, diluted 1 in 4 with 0.15 M saline and centrifuged in 100 ml aliquots at 150 g for 10 min at 4^{o}. The diluted plasma was removed and the cell pellet resuspended in the final drop of liquid. Seventy-five ml of deionized distilled water at 4^{o} was added, the tubes inverted and 25 ml of 0.6 M saline added immediately. The cells were centrifuged at 80 g for 10 min at 4^{o}, washed and pooled with Tyrode's buffer before being resuspended in 4 ml of PBS/HSA. The cells were then treated as for the bone marrow derived cells except that the gradient and fraction volumes were halved.

In experiments where basophils derived from the blood and bone marrow were compared, a sample of bone marrow was taken from each animal and treated as for the blood cells.

2.0 PREPARATION OF OTHER GUINEA PIG LEUCOCYTES

Eosinophils, neutrophils and macrophages were collected from the peritoneal cavity of Dunkin-Hartley guinea pigs of either sex. Bone marrow from untreated animals was also used. Cell counts were performed using white cell diluting fluid.

2.1 Eosinophil leucocytes

Eosinophils were obtained by the peritoneal lavage of animals which had received weekly intraperitoneal injections of horse serum (Wellcome Reagents Ltd., Beckenham, Kent) for six weeks (Kay, 1970b). The preparations were purified by centrifugation on cushions of sodium diatrizoate (Hypaque, Winthrop Laboratories, Surbiton-upon-Thames, Surrey) (Gleich and Loegering, 1973). A 45% Hypaque solution was diluted to the required density of 1.140 at 20^{o} with 0.15 M saline and EDTA, to give a final concentration of 0.1%, added.

2.2 Neutrophil leucocytes

Neutrophils were harvested from the peritoneal cavity, 3 hr after the intraperitoneal injection of 20 ml of 1 $mg.ml^{-1}$ glycogen (ex oyster pure AR, Koch-Light Laboratories Ltd., Colnbrook, Bucks.) (Jones et al, 1977).

2.3 Macrophages

Peritoneal macrophages were obtained by lavage 3 days after the intraperitoneal injection of 20 ml of 1 $mg.ml^{-1}$ glycogen and purified by centrifugation on Ficoll-Hypaque gradients (density: 1.077 $g.ml^{-1}$) (English and Anderson, 1974). The Ficoll-Hypaque used contained EDTA at a concentration of 0.1%.

2.4 Normal bone marrow

The bone marrow from an untreated guinea pig was collected as described in part 1.1 of this section. After a cell suspension was prepared, the cells were centrifuged at 100 g for 10 min at 4^{o} and resuspended in Tyrode's buffer at the required concentration.

3.0 PURIFICATION OF RAT PERITONEAL MAST CELLS

Mast cells were isolated by the method of Yurt *et al* (1977) with minor modifications. The cells of 20 Sprague-Dawley rats of either sex weighing 200-400 g were collected by lavage of the peritoneal cavity of each rat with 25-30 ml of Tyrode's buffer containing 0.1% gelatin and 10 units.ml^{-1} heparin. The cells were pooled and washed twice with Tyrode's buffer containing 0.1% gelatin and 30 $\mu g.ml^{-1}$ deoxyribonuclease 1 (Sigma Chemical Co., Poole, Dorset). One ml aliquots containing 3-7 x 10^7 cells in the same buffer were layered on 2 ml cushions of 22.5% metrizamide (Sigma Chemical Co., Poole, Dorset) and centrifuged at 85 g for 15 min at 20^{o}. The mast cells in the pellet and, if required, the interface containing mainly mononuclear cells, were washed with Tyrode's buffer and resuspended in the same buffer at the required concentration. The basophil stain, containing 10% guinea pig serum, was used to identify and count the mast cells.

SECTION III - MEASUREMENT AND MODULATION OF HISTIDINE AND HISTAMINE UPTAKE AND HISTAMINE SYNTHESIS

1.0 MEASUREMENT OF ^{14}C-HISTIDINE UPTAKE AND ITS CONVERSION TO ^{14}C-HISTAMINE

1.1 Basophil enriched cell suspensions

One ml volumes of basophil enriched cell suspensions containing 3-4 x 10^6 nucleated cells/ml in Tyrode's buffer were incubated in triplicate with 10 µl of L- [ring-2-^{14}C] histidine (10 µCi.ml^{-1} : 55 mCi.mmole^{-1}) (Radiochemical Centre, Amersham, Bucks.) in 15 ml plastic conical centrifuge tubes in a shaking water bath at 37°. The suspensions were diluted to 10 ml with ice-cold Tyrode's, centrifuged at 150 g for 5 min at 4°, the supernatants discarded and the cell pellets washed twice with 10 ml ice-cold Tyrode's. The washed cells were resuspended in 3 ml of Tyrode's buffer and 0.3 ml transferred to 6 ml scintillation vials. Three ml of Unisolve 1 (Koch-Light Laboratories Ltd., Colnbrook, Bucks.) were added to each vial and the amount of radioisotope present measured by liquid scintillation counting. All results were corrected by counting internal standards of known activity.

The remaining cells from each set of triplicates were pooled and centrifuged at 150 g for 5 min. The supernatant was decanted and the pellet of cells resuspended in the remaining solution before lysis by multiple (four times) freeze-thawing in an ethanol/dry ice bath. The resulting lysate was centrifuged at 250 g for 10 min at 4° and the supernatant applied to pre-coated cellulose sheets (Eastman Kodak, Kirby, Liverpool) scored into 1 cm wide strips.

The chromatograms were developed in ethanol:diethyl ether:NH_4OH:H_2O (8:10:1:2) until the solvent front had migrated 16 cm. Twenty µl of a mixture of authentic

L-histidine and histamine (1 mg.ml^{-1} of each) were used as markers, similarly chromatographed and identified by diazotized sulphanilic acid (Section I, part 3.2).

Areas of the lysate strips corresponding to the L-histidine (Rf 0.06 ± 0.004) and histamine (Rf 0.493 ± 0.01) containing regions were cut out and placed in vials containing 3 ml of toluene-based scintillation fluid (Section I, part 3.1). The amount of ^{14}C-histamine present in each strip was expressed as a percentage of the total radioactivity in that strip and the amount of histamine formed calculated from the total amount of histidine taken up by the cell suspensions.

1.2 Rat peritoneal mast cells

The uptake of ^{14}C-histidine and the amount of histamine formed was assessed by the method outlined above for basophil containing cell suspensions. In this case, however, 0.5 ml of purified mast cells containing 2-3 x 10^{6} cells.ml^{-1} were incubated with 5 µl of ^{14}C-histidine. After the final wash the cells were resuspended in 1 ml of Tyrode's buffer; 0.3 ml of this being used to measure the uptake and the rest subjected to chromatography.

1.3 Other cell types

Initial experiments were carried out as described in part 1.1. above. However, eosinophil enriched, neutrophil enriched, macrophage enriched and normal bone marrow cell suspensions were incapable of forming measurable amounts of ^{14}C-histamine from the incorporated ^{14}C-histidine. Therefore, following the final wash the cells were resuspended in 0.3 ml of Tyrode's buffer, all of which was used to assess ^{14}C-histidine uptake.

2.0 MEASUREMENT OF ^{14}C-HISTAMINE UPTAKE

2.1 Guinea pig derived cells

One ml samples of the cell suspensions were incubated in triplicate with 10 µl [ring-2-^{14}C] histamine dihydrochloride (10 µCi.ml^{-1} : 57 µCi.mmole^{-1}) (Radiochemical Centre, Amersham, Bucks.) in 15 ml plastic conical centrifuge tubes as described in part 1.1 for ^{14}C-histidine. Following the final wash the cells were resuspended in 0.3 ml Tyrode's buffer, all of which was transferred to the scintillation vials. Three ml of Unisolve 1 was added to each vial and the amount of ^{14}C-histamine present measured by liquid scintillation counting.

2.2 Rat peritoneal mast cells

The mast cell suspensions were treated as detailed above for guinea pig cells except that 0.5 ml of cells were incubated with 5 µl ^{14}C-histamine.

3.0 MEASUREMENT OF ^{14}C-LEUCINE UPTAKE

The uptake of ^{14}C-leucine was investigated with guinea pig cells only. One ml samples were incubated in triplicate with 10 µl L-$[1\text{-}^{14}C]$ leucine (10 $\mu Ci.ml^{-1}$: 59 $mCi.mmole^{-1}$) and treated as described for ^{14}C-histamine (Section III, part 2.1).

4.0 EFFECT OF AMINO ACIDS ON ^{14}C-HISTIDINE UPTAKE AND ^{14}C-HISTAMINE FORMATION

The following amino acids were used: L-alanine, L-arginine hydrochloride, L-aspartic acid, L-asparagine, L-cysteine hydrochloride, L-cystine, L-glutamic acid, L-glutamine, glycine, D-histidine hydrochloride, L-histidine, DL-isoleucine, DL-leucine, L-lysine monohydrochloride, DL-methionine, L-proline, DL-phenylalanine, L-serine, DL-threonine, DL-tryptophan, L-tyrosine and DL-valine (all from Sigma Chemical Co., Poole, Dorset).

0.9 ml samples of cells were added to 15 ml plastic conical centrifuge tubes containing 0.1 ml of amino acid or control solution (i.e. medium in which amino acid was dissolved) and 10 µl ^{14}C-histidine. The cell suspensions were incubated for 90 min at 37^{o} before being washed and treated as described in part 1.1 above.

Incubations containing cysteine also included dithiothreitol at a final concentration of 10 mmoles.l^{-1} to prevent cysteine oxidation. Tubes containing dithiothreitol were included as controls.

Cystine was insoluble at 10^{-2} moles.l^{-1} and, therefore, it was dissolved at 1.1×10^{-3} moles.l^{-1}. In this case, 0.9 ml of cystine or control solution was added to the centrifuge tubes and 0.1 ml of a ten times concentrated cell suspension was added.

5.0 EFFECT OF METABOLIC INHIBITORS, PHARMACOLOGICAL MEDIATORS AND DRUGS ON ^{14}C-HISTIDINE AND ^{14}C-HISTAMINE UPTAKE AND ^{14}C-HISTAMINE FORMATION

The materials were obtained as follows. Metabolic inhibitors: actinomycin D, antimycin A, colchicine, cycloheximide, cytochalasin B, 2-deoxyglucose, 2,4-dinitrophenol, iodoacetamide, mitomycin C, ouabain and sodium fluoride (Sigma Chemical Co., Poole, Dorset); 4, bromo-3-hydroxybenzylosyamine dihydrogen phosphate (NSD 1055) (Sandev Ltd., Harlow, Essex).

Pharmacological mediators: histamine acid phosphate (BDH Chemicals Ltd., Poole, Dorset); bradykinin triacetate and 5-hydroxytryptamine creatinine sulphate complex (Sigma Chemical Co., Poole, Dorset); ECF-A tetrapeptides were a gift from Dr. R. Camble (ICI Ltd., Pharmaceuticals Division, Alderley Park, Macclesfield, Cheshire); prostaglandins E_1, E_2 and $F_{2\alpha}$ were a gift from Dr. J. Pike (Upjohn Company, Kalamazoo, U.S.A.).

Drugs: hydrocortisone, indomethacin and aspirin were a gift from Dr. F.R. Mangan (Beecham Pharmaceuticals, Research Division, Harlow, Essex); disodium cromoglycate ("Intal") was a gift from Fisons Pharmaceuticals Ltd., Loughborough, Leics.

Imidazoleacetic acid hydrochloride (Sigma Chemical Co., Poole, Dorset); 1,4-methylhistamine dihydrochloride, N-acetylhistamine and 1-methyl-4-imidazoleacetic acid (Calbiochem Ltd., San Diego, California, U.S.A.); mepyramine maleate (May and Baker Ltd., Dagenham, Essex); chlorpheniramine (Allen and Hanburys Ltd., London); burimamide, metiamide, 2-(2-aminoethyl) thiazole dihydrochloride, 4-methylhistamine dihydrochloride,

and S-[3-(N,N-dimethylamino) propyl] isothiourea dihydrochloride ("Dimaprit") were a gift from Smith, Kline and French Laboratories Ltd., Welwyn Garden City, Herts.

All these compounds were dissolved in Tyrode's buffer except for cytochalasin B which was dissolved in dimethyl sulphoxide at 10^{-3} moles.l^{-1} and hydrocortisone, indomethacin and aspirin which were dissolved in ethanol at 10^{-2} moles.l^{-1}. These were then diluted to the required concentration with Tyrode's buffer before use and compared to the appropriate controls.

0.9 ml cell suspensions were added to 15 ml plastic conical centrifuge tubes containing 0.1 ml of agent or control solution and preincubated in a shaking water bath at 37° for 40 min. Ten µl of ^{14}C-histidine or ^{14}C-histamine were then added and the cells incubated for a further 90 min before being treated as described above (parts 1.1 and 2.1).

5.1 Effect of sodium ions

To assess the effect of sodium ions, basophil enriched cell suspensions were washed five times with modified PBS or choline substituted modified PBS (Section I, part 1.4). The cells were resuspended in the same buffers at 3-5 x 10^6 nucleated cells/ml, and incubated with ^{14}C-histidine or ^{14}C-histamine as described above (part 1.1 and 2.1).

5.2 Time courses

0.9 ml of cell suspensions in triplicate were incubated with 0.1 ml of ouabain (10^{-4} moles.l^{-1}), antimycin A (10^{-5} moles.l^{-1}), histamine (10^{-2} moles.l^{-1}) or diluent control for 40 min at 37° in a shaking water bath and then incubated for

various times with 10 µl ^{14}C-histidine. The cells were washed and treated as described above (part 1.1).

To study the effect of increased exposure to histamine, 0.9 ml of cells were incubated with 0.1 ml histamine (10^{-2} moles.l^{-1}) or diluent control for various times before the addition of ^{14}C-histidine and then treated as for other time courses. In experiments where the histamine was removed before the addition of the ^{14}C-histidine the cells were washed twice with 10 ml of ice-cold Tyrode's buffer. After the final wash the cells were resuspended in 1 ml of the same buffer for incubation with ^{14}C-histidine as for other time courses.

5.3 Effect of histamine antagonists on the enhancement of histidine uptake and histamine formation

0.8 ml of basophil enriched cell suspensions were added to a 15 ml plastic conical centrifuge tube containing 0.1 ml antagonist for 20 min at 37° in a shaking water bath. 0.1 ml of histamine (10^{-2} moles.l^{-1}) or diluent control was added for a further 20 min and then 10 µl ^{14}C-histidine. The cell suspensions were incubated for 90 min and treated as described above (part 1.1).

The effect of the antagonists was expressed as the percentage decrease of enhancement:

% decrease of enhancement =

$$100 - \left(\frac{\text{(value for antagonist + histamine)} - \text{value for antagonist}}{\text{(value for histamine)} - \text{value for antagonist}} \times 100 \right)$$

6.0 EFFECT OF ANAPHYLAXIS

6.1 Endogenous histamine content

Total endogenous histamine was extracted from guinea pig basophil enriched cell suspensions and purified rat mast cells by multiple (four times) freeze-thawing in an ethanol/dry ice bath. Histamine was measured by bioassay using guinea pig ileum suspended in oxygenated atropinized Tyrode's solution (0.05 μ moles atropine.l^{-1}) in a 2 ml capacity organ bath maintained at 37° by a continuous flow water jacket (Brocklehurst, 1960).

6.2 Measurement of anaphylactic histamine release

0.9 ml of basophil enriched cell suspensions, in duplicate, were incubated at 37° for 15 min prior to the addition of 0.1 ml antigen (Section I, part 3.3) or control, which were also incubated at 37°, for a further 15 min. The cells were centrifuged at 150 g for 5 min at 4° and the supernatant removed. The pelletted cells were resuspended in 1 ml Tyrode's buffer and lysed by multiple (four times) freeze-thawing to release the residual histamine. The released and residual histamine were measured by bioassay as described above for endogenous histamine content.

The amount of histamine released was expressed as a percentage of the endogenous content prior to challenge:

$$\% \text{ histamine released} = \frac{\text{Amount of histamine released}}{\text{Amount of histamine released} + \text{Amount of residual histamine}} \times 100$$

6.3 Effect of anaphylactic histamine release on the uptake of ^{14}C-histidine and ^{14}C-histamine formation

Sheep's blood treated animals were used as the source of

the basophils and sheep's blood cell lysate as the antigen. A set of tubes were included to measure the amount of histamine released as a control to test the cells and antigen.

Immediately after challenge: cells were challenged in triplicate with various dilutions of antigen as described in part 6.2. The cells were washed twice with Tyrode's buffer and then resuspended in 1 ml of the same buffer for incubation with ^{14}C-histidine for 90 min as described above (part 1.1).

Twenty-four hours after challenge: 4.5 ml of pre-warmed basophil enriched cell suspensions in Tyrode's buffer were challenged with 0.4 ml of antigen or control solution for 15 min at 37^{o} in sterile plastic universal containers. The cells were washed twice with the same buffer and resuspended in 10 ml Eagle's minimal essential medium without histidine (Section I, part 1.5) containing 10% fresh guinea pig serum and incubated at 37^{o} for 24 hr with continual gentle mixing. The cells were then washed with 20 ml Tyrode's buffer, re-suspended in 4 ml of the same buffer and a cell count performed. One ml aliquots were added in triplicate to 15 ml plastic conical centrifuge tubes and incubated with ^{14}C-histidine for 90 min as described above (part 1.1).

Time course: 22.5 ml of pre-warmed cell suspensions rich in basophils were challenged with 2.5 ml antigen or control solution as detailed above (24 hr after challenge). After the washes they were resuspended in 25 ml Tyrode's buffer at 37^{o}, a cell count performed and 0.25 ml of ^{14}C-histidine added. At each time point, triplicate 1 ml samples were removed and the amount of ^{14}C-histidine incorporated and histamine formed determined as described above (part 1.1).

7.0 EFFECT OF VARIOUS CELL TYPES ON THE UPTAKE OF ^{14}C-HISTIDINE AND ^{14}C-HISTAMINE FORMATION BY BASOPHIL ENRICHED CELL SUSPENSIONS

7.1 On unchallenged basophils

After 40 min incubation: centrifuge tubes were set up in triplicate containing 0.5 ml basophil enriched cell suspension, 8-10 x 10^6 nucleated cells.ml^{-1}, and 0.5 ml of the cell type being investigated, at the highest concentration possible (in practice 10-12 x 10^6 cells.ml^{-1}) or 0.5 ml of either cell type and 0.5 ml Tyrode's buffer. The cells were mixed and incubated at 37^o for 40 min before the addition of ^{14}C-histidine for a further 90 min. The amount of ^{14}C-histidine incorporated and ^{14}C-histamine formed was determined as described above (part 1.1).

In two sets of experiments the eosinophils were incubated with horse serum for 15 min at 37^o and washed twice with Tyrode's buffer prior to mixing with the basophil enriched cell suspensions or Tyrode's.

After 18 hr incubation: 3 ml of basophil containing cell suspensions plus 2 ml of eosinophil enriched cell suspensions or the two cell populations alone were added to sterile plastic universal containers. The cells were centrifuged at 100 g for 10 min at 4^o, the pelletted cells resuspended in 10 ml Eagle's minimal essential medium without histidine containing 10% fresh guinea pig serum and incubated at 37^o for 18 hr with continuous gentle mixing. The uptake of ^{14}C-histidine and ^{14}C-histamine formation was assessed as in part 6.3 (24 hr after challenge).

7.2 On challenged basophils

Three ml of a basophil containing cell suspension plus 2 ml of a cell suspension rich in eosinophils, or each cell type alone (concentrations as in part 7.1), were added to a sterile plastic universal container. The cells were centrifuged at 100 g for 10 min at 4^{o} and resuspended in 4.5 ml Tyrode's buffer. After equilibration for 40 min in a 37^{o} shaking water bath they were challenged with antigen or control solution and treated as described in part 6.3 (24 hr after challenge) except that they were cultured for only 18 hr.

8.0 EFFECT OF EOSINOPHIL DERIVED PRODUCTS ON ^{14}C-HISTIDINE UPTAKE AND ^{14}C-HISTAMINE FORMATION

Purified human eosinophil cationic protein and lysozyme were a gift from Dr. Per Venge, University of Uppsala, Sweden.

The guinea pig eosinophil granule extract was prepared by the method of Gleich et al (1973). Eosinophils were obtained as described in Section II, part 2.1. The cells from five guinea pigs were pooled, washed twice in Tyrode's buffer and resuspended in 10 ml 0.34 moles.l^{-1} sucrose. The cells were lysed by numerous vigorous passages out of a 20 ml syringe. The resultant suspension was centrifuged at 400 g for 10 min at 4^{o} to remove cell debris and any intact cells. The supernatant was then subjected to 5000 g for 20 min at 4^{o} and the pelletted granules solubilized with 0.5 ml 0.01 moles.l^{-1} HCl.

The effect of the purified cationic proteins, lysozyme and the crude granule preparation on ^{14}C-histidine uptake and ^{14}C-histamine formation was studied as in part 5.0 above.

9.0 ANAPHYLACTIC RELEASE OF ^{14}C-HISTAMINE AND ^{14}C-HISTIDINE

Cells were incubated with ^{14}C-histidine in the presence or absence of NSD 1055 as detailed above (part 5.0). After the final wash one triplicate of each treatment was processed as described for the determination of ^{14}C-histidine uptake and ^{14}C-histamine formation. The rest were resuspended in 0.45 ml of Tyrode's buffer and challenged with 0.05 ml antigen or control solution, after equilibration at 37°, for 15 min. The cells were centrifuged at 150 g for 5 min at 4° and the supernatants removed. 0.05 ml of this were transferred to a scintillation vial to which was added 0.25 ml distilled water and 3 ml Unisolve 1. The amount of radioisotope present was determined by liquid scintillation counting. A further 0.1 ml was stopped on a chromatogram scored in 2 cm strips and processed as described in Section III, part 1.1 to determine what proportion of the radioisotope present corresponded to ^{14}C-histidine and ^{14}C-histamine.

The amount of ^{14}C-histamine released was expressed as a percentage of that present within the cells prior to challenge.

The amount of ^{14}C-histidine released was expressed as a percentage of that present in the supernatant of cells which were incubated with control solution instead of antigen.

10.0 STATISTICS

Measurements from triplicate incubations for uptake studies and single values for ^{14}C-histamine formation determinations from at least three separate experiments were pooled, the mean and standard errors calculated and the observations compared using the student's t-test. Such pooling was considered to be statistically valid since in the present study the variation within each experiment was similar to the variations between individual experiments as previously observed (Clark *et al*, 1975).

CHAPTER IV - RESULTS

SECTION I - HISTIDINE AND HISTAMINE UPTAKE AND NEW HISTAMINE FORMATION BY BASOPHILS: OPTIMAL CONDITIONS, SPECIFICITY AND COMPARISON WITH OTHER LEUCOCYTES

1.0 INTRODUCTION

The basophil leucocyte is known to participate in both immediate and delayed type hypersensitivity reactions (Ishizaka and Ishizaka, 1971; Richerson *et al*, 1970). Until recently there were difficulties in the study of basophil function and the elucidation of its role in the immune response because of the paucity of numbers that normally circulate in the peripheral blood. Although partial purification of both human and guinea pig basophils has been achieved by a number of workers (Sampson and Archer, 1967; Pruzansky and Patterson, 1970; Wilson and Coombs, 1971), the first reliable method for obtaining large numbers of purified basophils was not developed until more recently (Dvorak *et al*, 1974). Basophil-enriched cell suspensions obtained by a slight modification of this method have been used to study several biological variables, viz. the uptake of ^{14}C-histidine and ^{14}C-histamine and the formation of ^{14}C-histamine *de novo*, which may be relevant to histamine synthesis and storage.

This section describes the attempts which were made to study the mechanism of transmembrane movement of histamine and its precursor, histidine, by determining some of the factors which may influence their uptake. To this end the effects of variations in the time of incubation, extracellular concentration, temperature and pH and inter-amino acid competition were investigated. Also, attempts were made to compare other leucocytes, with basophils, in terms of histidine and histamine uptake and histamine synthesis.

2.0 PURIFICATION OF BONE MARROW BASOPHILS

The mean purification data for 417 sheep's blood treated, 52 ovalbumin treated and 21 stock guinea pigs is shown in Table I.

The bone marrow from guinea pigs which had received 12 ip. injections of sheep's blood contained 77.2 ± 1.6 x 10^6 (mean ± 1 S.E.) basophils which accounted for 7.9 ± 0.2% of the total nucleated cells. After treatment with ex-carboxyl iron the basophil yield was 97.4 ± 2.6% whereas only 73.5 ± 1.3% of the total nucleated cells were recovered. The removal of phagocytic cells resulted in a cell suspension containing 5-24% (mean 10.2) basophils.

A typical distribution following centrifugation on a Ficoll gradient is shown in the upper part of Fig. 6. The total nucleated cell peak, at 6.0-7.0% Ficoll (1.025-1.027 $g.ml^{-1}$), included numerous lymphocytes and a variety of mature and immature nucleated forms. The basophil peak, at 8.0-8.7% Ficoll (1.030-1.032 $g.ml^{-1}$) included basophils at various stages of development and a heterogeneous mixture of immature myeloid and erythroid cells. In general 60-100 x 10^6 basophils, the majority mature, were present in the pooled Ficoll fractions from two sheep's blood treated guinea pigs. A few neutrophils (about 1-3%) and eosinophils (about 2-5%) were also present but erythrocyte contamination was relatively negligible, the ratio of nucleated cells to erythrocytes being approximately 3:1. Using sheep's blood as the antigen, an overall basophil purification of 2.5-8.0 (mean 3.8) fold was achieved, whilst with ovalbumin the results were similar (2.5-11.0 fold, mean 4.9).

	SHEEP'S BLOOD PRIMED	OVALBUMIN PRIMED	UNTREATED
Number of Animals	417	52	21
Total Nucleated Cells	$990.2 \pm 14.5 \times 10^6$	$794.0 \pm 24.9 \times 10^6$	$735.5 \pm 49.9 \times 10^6$
Basophils	$77.2 \pm 1.6 \times 10^6$	$39.9 \pm 3.1 \times 10^6$	$8.0 \pm 1.0 \times 10^6$
Basophils (%)	7.9 ± 0.2	5.0 ± 0.3	1.1 ± 0.1
Basophils Post Phagocytosis (%)	10.2 ± 0.3	7.0 ± 0.6	-
Basophils Peak Fraction (%)	29.7 ± 0.6	24.6 ± 1.7	-

TABLE I

Basophil purification data from guinea pigs which had received 12 daily intraperitoneal injections of sheep's blood or ovalbumin or no treatment.

The leucocytes obtained from the Ficoll gradients were greater than 98% viable (as judged by trypan blue dye exclusion) were capable of *in vitro* migration to complement activated guinea pig serum (Jones and Kay, 1974) and released histamine when challenged by antigen.

A preparation of purified guinea pig bone marrow basophils is shown in plate I.

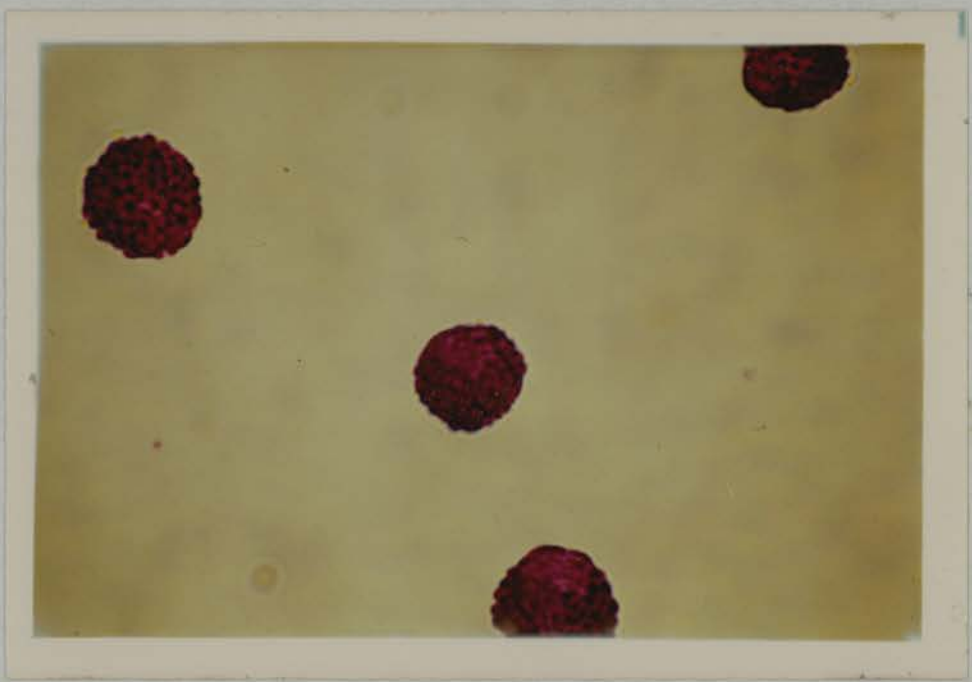

Plate I

Preparation of guinea pig bone marrow basophil leucocytes. (May Grunwald/Giemsa, x 2000 magnification)

3.0 DETERMINATION OF OPTIMUM CONDITIONS

Varying numbers of basophil-enriched cell suspensions were incubated with 10 µl ^{14}C-histidine for 90 min and the amounts of ^{14}C-histidine taken up and ^{14}C-histamine formed determined (Fig. 1). There was direct proportionality between the number of cells and the amount of ^{14}C-histidine incorporated and ^{14}C-histamine synthesized. Therefore, the number of cells used in all other experiments, 3-5 x 10^6 nucleated cells, did not appear to be a limiting factor of any significance.

The efficiency of the washing procedure employed is demonstrated in Table II. There was no significant decrease in the amount of radioisotope present in the supernatant after the second wash. The amount of ^{14}C-labelled histidine and histamine present in the third and subsequent washes was negligible compared to that associated with the cells. In all further experiments presented the cells were, therefore, washed twice and the amounts of ^{14}C-histidine or ^{14}C-histamine present in the final cell suspensions were assumed to represent that associated with the cells.

3.1 Time course

The variation in the uptake of ^{14}C-histidine and ^{14}C-histamine and the amount of ^{14}C-histamine formed with time is shown in Fig. 2. There was a rapid initial uptake of ^{14}C-histidine which slowed down after about 60 min and reached a constant level at approximately 180 min. By 240 min 10% of the added ^{14}C-histidine had been incorporated. The time course of ^{14}C-histamine formation did not exhibit a comparable initial rapid rate but there was a progressive

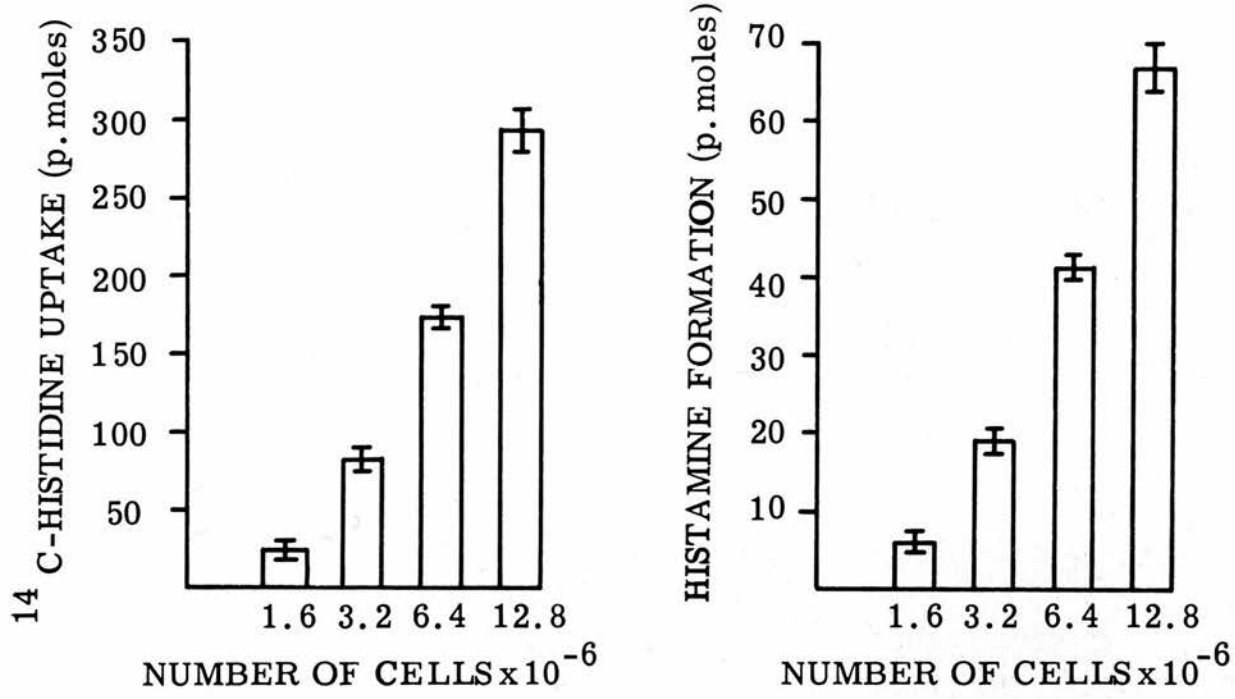

Fig. 1

The uptake of ^{14}C-histidine and the formation of ^{14}C-histamine by various concentrations of cells rich in basophils. Each column represents the mean ± 1 S.E. of 1 experiment, in triplicate.

	INCUBATION WITH ^{14}C-HISTIDINE (d.p.m.)			INCUBATION WITH ^{14}C-HISTAMINE (d.p.m.)	
1st Spin	171511	169653	173679	198179	204616
1st Wash	6008	6417	6850	4950	4500
2nd Wash	1919	1383	1410	320	325
3rd Wash	1506	1337	940	140	65
4th Wash	1491	1327	1010	145	59
5th Wash	995	1200	960	-	-
Cells	43068	49500	37510	710	740
Total Recovered	226498	230817	222359	204444	210305
% Recovered	102.0	104.0	100.2	92.1	94.7

TABLE II

The distribution of radioisotope, expressed as disintegrations per minute (d.p.m.), in the washing supernatants and cell pellet of basophil-enriched cell suspensions after incubation with 0.1 μCi (222,000 d.p.m.) ^{14}C-histidine or ^{14}C-histamine for 90 min.

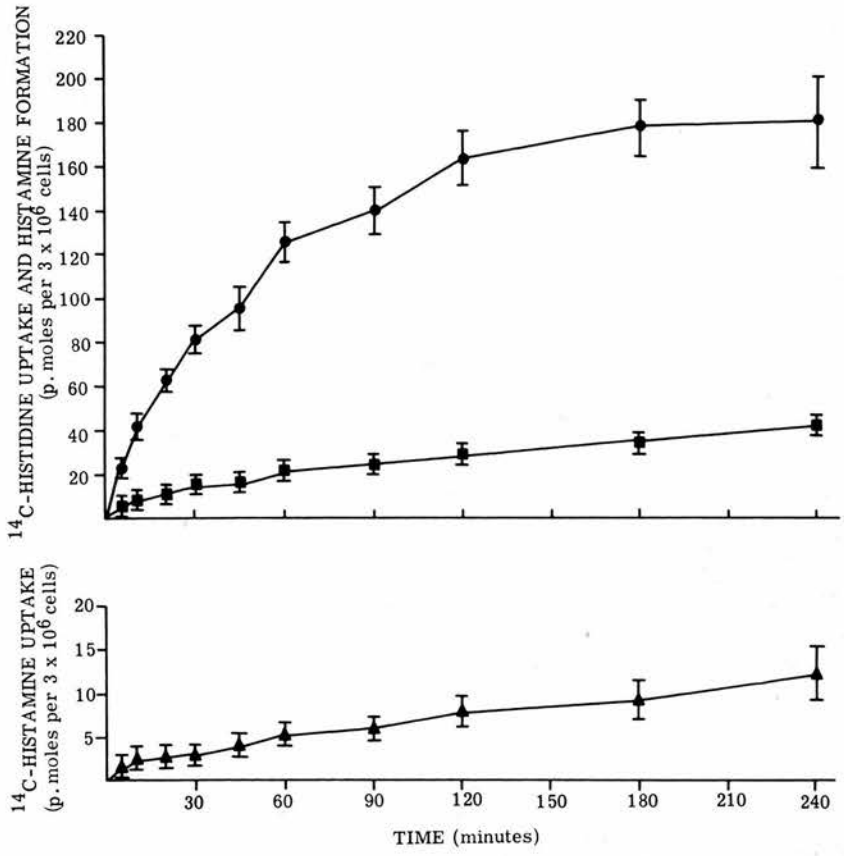

Fig. 2

Time course of uptake of ^{14}C-histidine (●—●) and ^{14}C-histamine (▲ — ▲) and the amounts of ^{14}C-histamine formed (■ — ■) by basophil-enriched cell suspensions. Each point represents the mean ± 1 S.E. of 8 experiments for ^{14}C-histidine uptake and ^{14}C-histamine formation and 3 experiments for ^{14}C-histamine uptake.

build-up of newly synthesized ^{14}C-histamine. This gradual increase persisted for the duration of the experiment at which time ^{14}C-histamine accounted for 24% of the incorporated radioisotope.

In contrast to ^{14}C-histidine, the amount of ^{14}C-histamine incorporated was comparatively small. Thus even after the termination of the experiment at 240 min only 0.7% of the added ^{14}C-histamine had been taken up.

In further experiments a time of 90 min was chosen since this gave an appreciable uptake compared to "background" values. Also, in experiments with metabolic inhibitors and histamine receptor antagonists (see below) there was a slight loss of cell viability when incubations were performed for longer periods of time.

3.2 Influence of extracellular histidine and histamine concentration

The results of histidine and histamine uptake and histamine formation measured as a function of extracellular histidine and histamine concentration are shown in Figs. 3 and 3A. There was a rapid increase in the amount of histidine incorporated as the concentration of histidine was raised and over the concentration range used (0.18-1000 μ.moles.l^{-1}) saturation was not reached. At the lowest concentration used, 0.45 μ.moles.l^{-1}, 11% of the added ^{14}C-histidine was incorporated while only 0.8% was taken up when the extracellular concentration was 1 m.mole.l^{-1}. The percentage of the ^{14}C-histidine taken up which was converted to ^{14}C-histamine decreased with increasing extracellular histidine concentration. The absolute amounts of

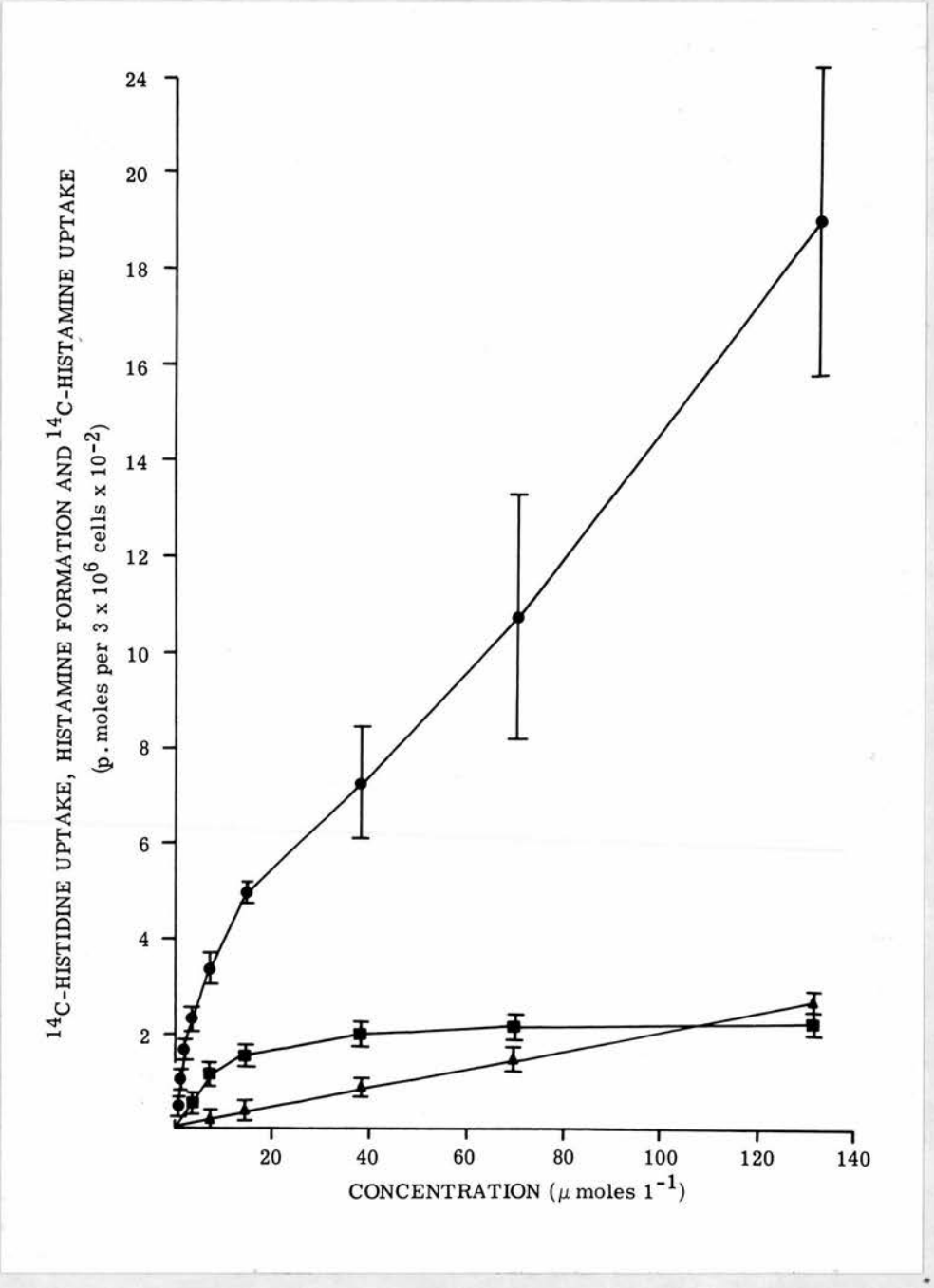

Fig. 3

The effect of increasing the extracellular concentrations of ^{14}C-histidine on ^{14}C-histidine uptake (● — ●) and ^{14}C-histamine formation (■ — ■) and of increasing the extracellular concentration of ^{14}C-histamine on ^{14}C-histamine uptake (▲ — ▲). The results are the mean ± 1 S.E. of 4 experiments.

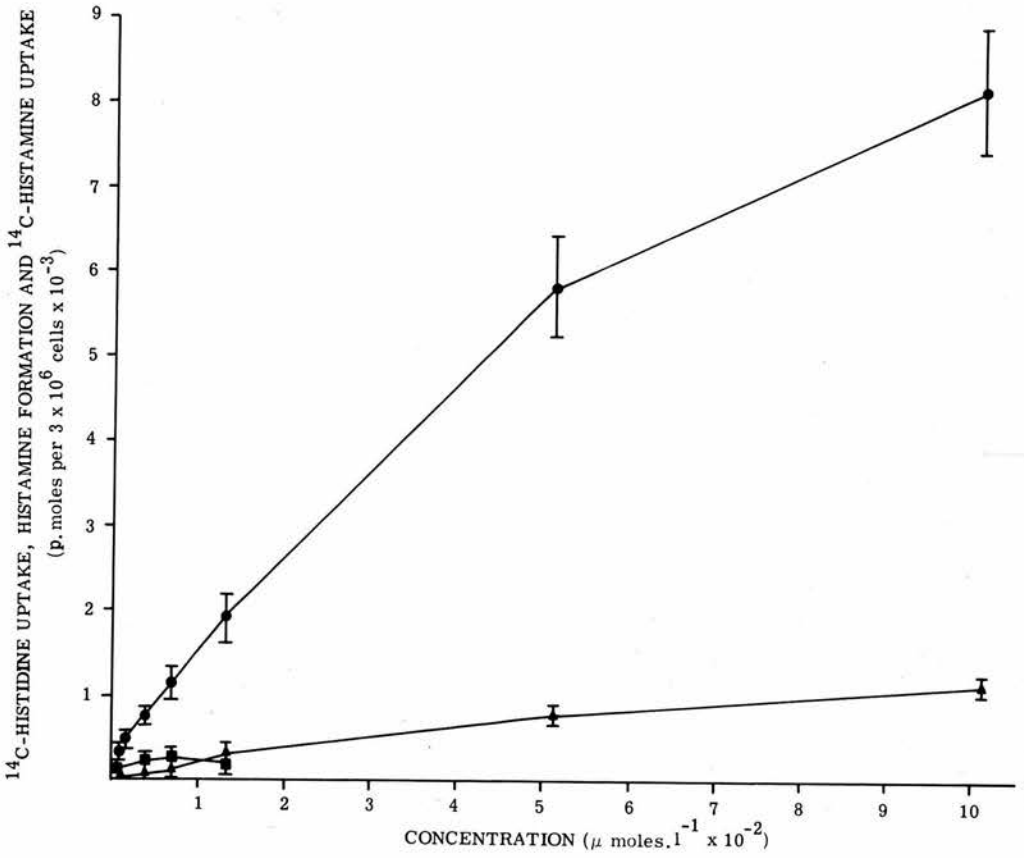

Fig. 3A

The effect of increasing the extracellular concentrations of ^{14}C-histidine on ^{14}C-histidine uptake (● — ●) and ^{14}C-histamine formation (■ — ■) and of increasing the extracellular concentration of ^{14}C-histamine on ^{14}C-histamine uptake (▲ — ▲). The results are the mean ± 1 S.E. of 4 experiments.

^{14}C-histamine formed reached a plateau at an extracellular histidine concentration of 70 μ.moles.l^{-1}. It should be noted that histidine concentrations above 7.2 μ.moles.l^{-1} were obtained by the addition of increasing amounts of unlabelled histidine. This reduced the specific activity to such an extent that due to the limitations of the techniques used, determination of histamine formation was not possible at histidine concentrations above 130 μ.moles.l^{-1}.

There was a progressive increase in the amount of histamine taken up as its extracellular concentration was raised. The percentage of added histamine incorporated did not vary substantially (0.26-0.11%) over the concentration range used (0.18-1000 μ.moles.l^{-1}).

Concentrations of 1.8 μ.moles.l^{-1} histidine and histamine were used in further studies since these resulted in incorporation significantly above "background" but kept the quantity of radioisotope used to a minimum.

3.3 Effect of varying the incubation temperature

The uptake of ^{14}C-histidine showed marked temperature dependence, being maximal at 37°, whereas ^{14}C-histamine uptake and formation, although dependent on temperature, continued to increase up to 43° (Fig. 4). There was low but significant uptake of ^{14}C-histidine at 0° which increased steadily to peak at 37°. ^{14}C-histamine formation increased less rapidly but did not show the same decrease that was found with ^{14}C-histidine uptake above 37°. There was very little uptake of ^{14}C-histamine below 30° but between 30 and 43° a very rapid increase occurred.

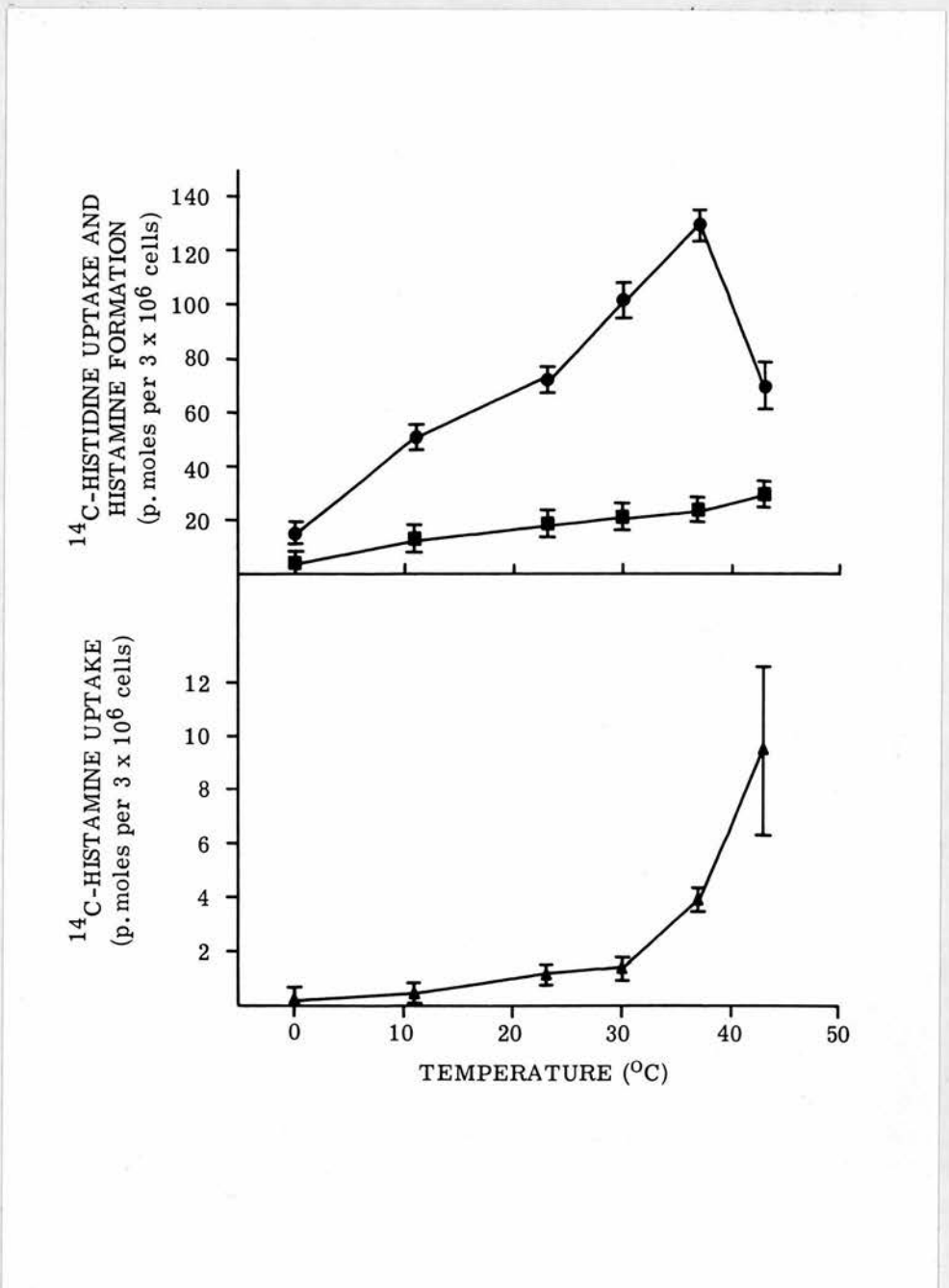

Fig. 4

The effect of varying the incubation temperature on the uptake of ^{14}C-histidine (● — ●) and ^{14}C-histamine (▲ — ▲) and the amount of histamine formed (■ — ■). Each point represents the mean ± 1 S.E. of 4 experiments where cells were incubated with ^{14}C-histidine and 3 experiments where cells were incubated with ^{14}C-histamine.

Membranes undergo phases change at various temperatures associated with an increase in fluidity. The rapid increase in ^{14}C-histamine uptake above 30^{o} may have been due to these changes whereas ^{14}C-histidine uptake did not seem to be affected to the same extent.

3.4 Effect of pH

The uptake of ^{14}C-histidine and ^{14}C-histamine and ^{14}C-histamine formation over the pH range 6.5-8.0 is shown in Fig. 5. There was no significant variation in ^{14}C-histidine uptake or ^{14}C-histamine formation under these conditions. However, the amount of ^{14}C-histamine incorporated varied at either end of the pH range when compared to physiological values (pH 7.2-7.4). There was a significant difference ($p < 0.05$) between the uptake at pH 6.5 and 8.0.

All further experiments were carried out in buffer at pH 7.25-7.4 and all solutions were adjusted to the same pH as the cell suspensions to which they were added.

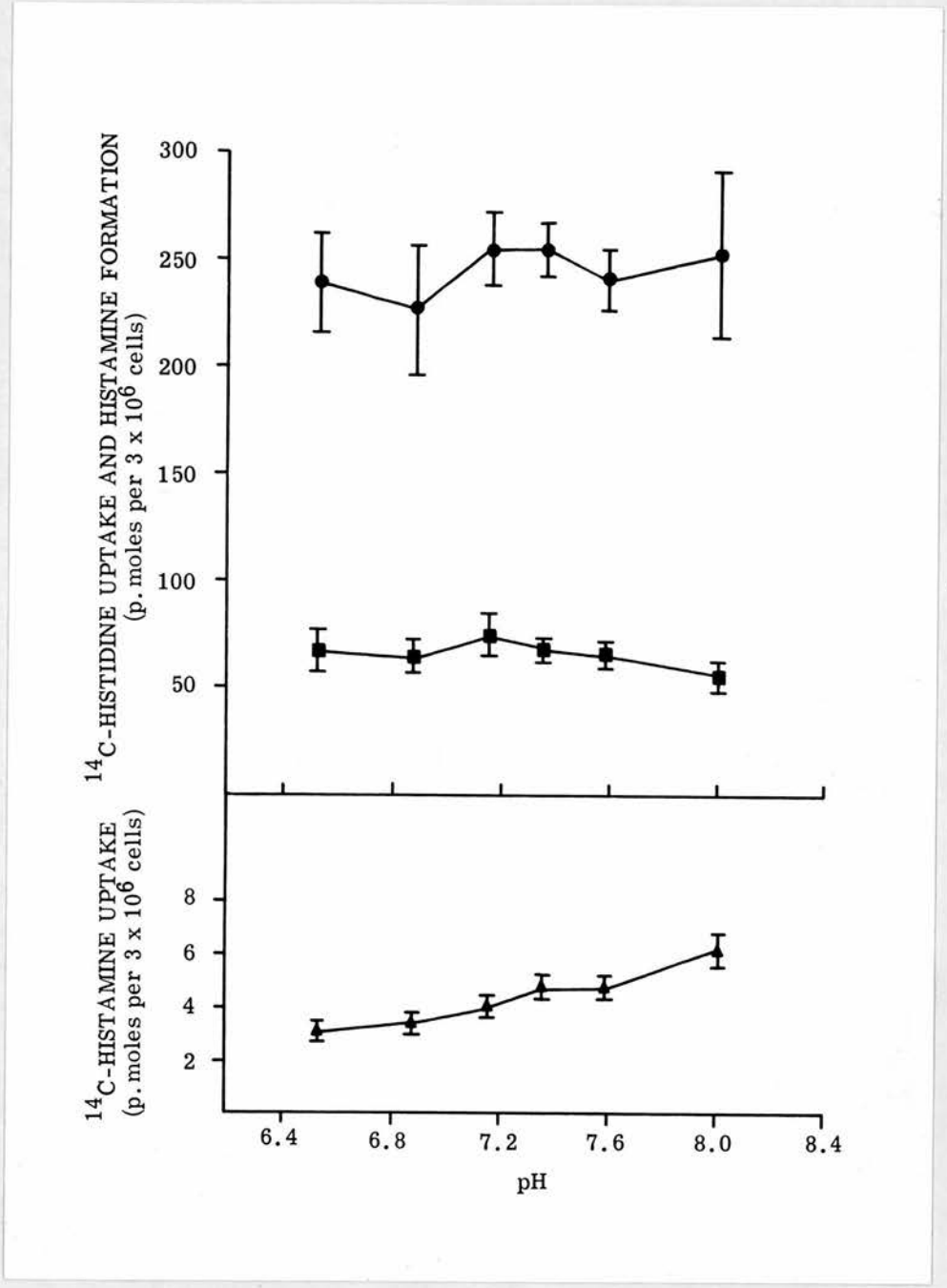

Fig. 5

The effect of varying the incubation pH on the uptake of ^{14}C-histidine (● — ●) and ^{14}C-histamine (▲ — ▲) and the amount of histamine formed (■ — ■). Each point represents the mean of ± 1S.E. of 5 experiments where cells were incubated with ^{14}C-histidine and 4 experiments where cells were incubated with ^{14}C-histamine.

4.0 SPECIFICITY OF HISTIDINE AND HISTAMINE UPTAKE AND HISTAMINE FORMATION

The specificity of the uptake of histidine and histamine and histamine synthesis was investigated using several approaches including (i) populations of varying basophil content, (ii) different cell types and (iii) inhibition studies with other amino acids.

4.1 Correlation with basophil content

Those fractions, from a Ficoll gradient, which contained the greatest number of basophils corresponded to (i) the peaks of maximal incorporation of ^{14}C-histidine, (ii) the maximal amount of ^{14}C-histamine synthesis *de novo* and (iii) the highest concentration of "extractable" histamine (Fig. 6). In contrast, ^{14}C-histamine uptake was minimal in all the fractions and showed no relation to their basophil content.

The percentage of basophils in cell suspensions from 80 different guinea pigs (each batch of cells being the pool from two animals) was compared with their capacity to incorporate either ^{14}C-histidine or ^{14}C-histamine or synthesize new histamine (Fig. 7). The uptake of ^{14}C-histidine and ^{14}C-histamine formation was directly proportional to the percentage of basophils in these populations ($p < 0.001$). In contrast, there was no correlation with ^{14}C-histamine uptake.

4.2 Different cell types

Differences in ^{14}C-histidine, ^{14}C-histamine and ^{14}C-leucine uptake between basophils and other leucocytes are shown in Fig. 8.

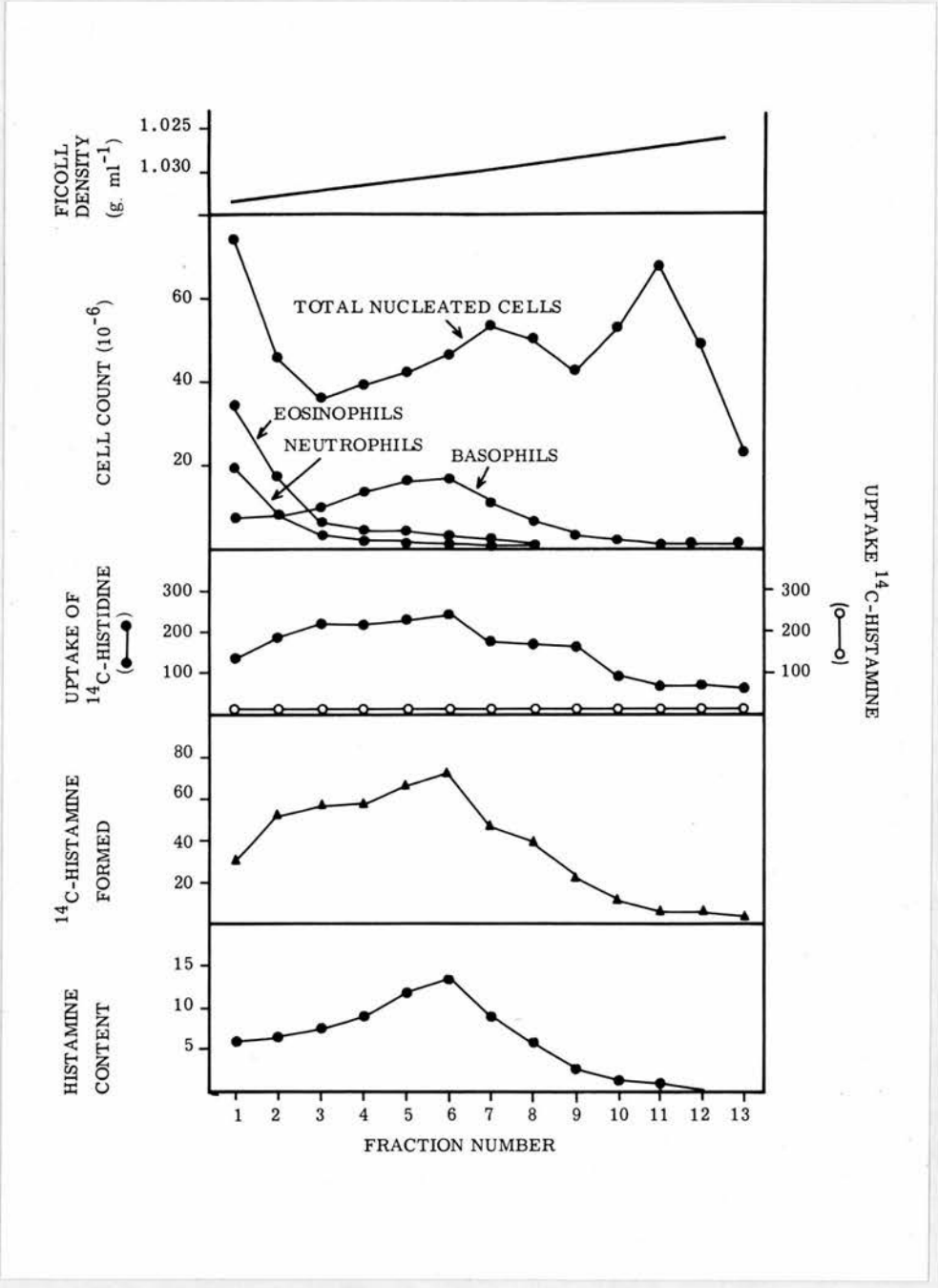

Fig. 6

Separation of guinea pig bone marrow cells by a continuous Ficoll gradient. The uptake of ^{14}C-histidine and ^{14}C-histamine and the amount of ^{14}C-histamine formed are expressed as p.moles per 3×10^6 nucleated cells. The histamine content is expressed as the number of µg histamine present per fraction.

The figure is a representative experiment which has been repeated 3 times.

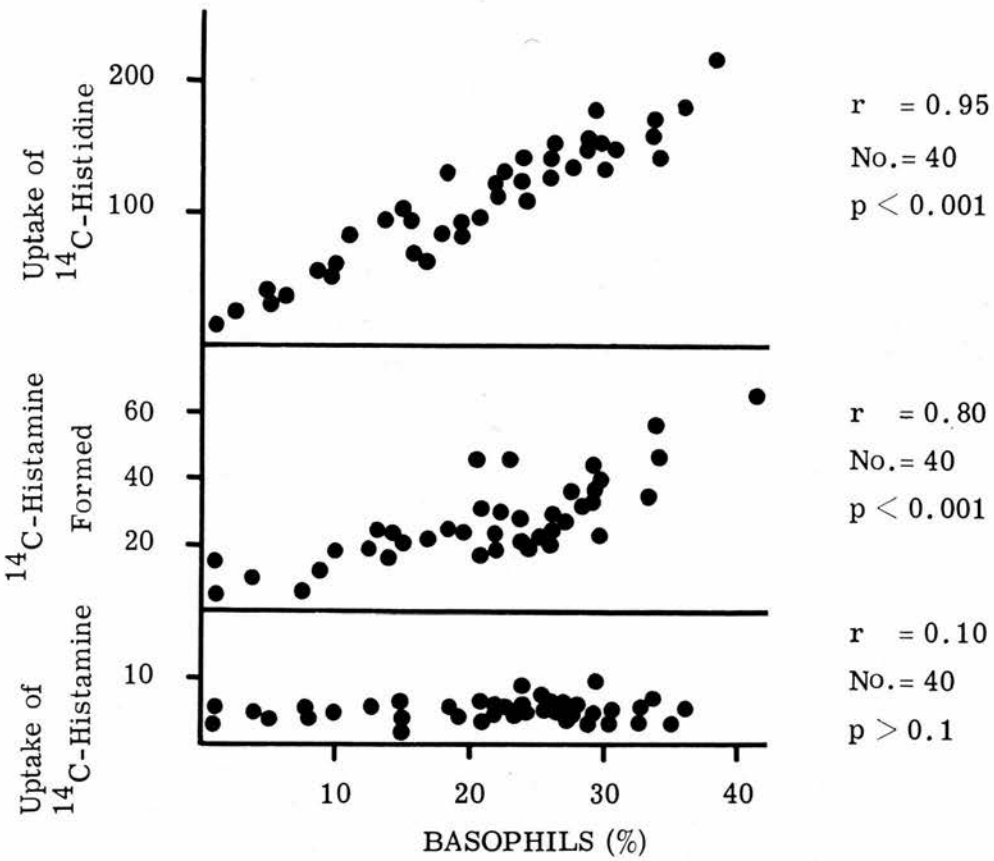

Fig. 7

Comparison of the uptake of ^{14}C-histidine and ^{14}C-histamine and the amounts of ^{14}C-histamine formed with cell suspensions containing varying percentages of basophils. The uptake of ^{14}C-histidine and ^{14}C-histamine and the amount of ^{14}C-histamine formed are expressed as p.moles per 3 x 10^6 cells.

The uptake of ^{14}C-histidine by cell suspensions enriched with eosinophils (88%), neutrophils (95%) and macrophages (92%) and normal bone marrow cells was 18.2%, 22.0%, 40.9% and 34.3% (respectively) of that observed in basophil-enriched suspensions. Since on average the basophil-enriched cell suspensions contained only 30% basophils the data suggests that histidine uptake by this cell type was highly preferential. The uptake of ^{14}C-histamine by all cell types studied was extremely low compared to ^{14}C-histidine uptake. Basophil-enriched cell suspensions incorporated 35.9%, 231.5%, 76.7% and 307.7% more ^{14}C-histamine than eosinophil-rich, neutrophil-rich, macrophage-rich and normal bone marrow cell suspensions, respectively.

^{14}C-leucine was most avidly taken up by the cell suspensions enriched with basophils. Under similar conditions all the cell types tested, apart from the eosinophil, incorporated a greater amount of ^{14}C-leucine than ^{14}C-histidine. However, eosinophil-rich, neutrophil-rich, macrophage-rich and normal bone marrow cell suspensions incorporated only 12.9%, 30.8%, 74.5% and 37.6% (respectively) of the ^{14}C-leucine taken up by basophil-rich preparations.

^{14}C-histamine formation was only detected in the basophil-rich preparations.

The uptake of ^{14}C-histidine and ^{14}C-histamine and ^{14}C-histamine formation was also compared in basophil-enriched cell suspensions derived from the peripheral blood and bone marrow of the same animals (Table III). Although the percentage of basophils in each preparation was approximately

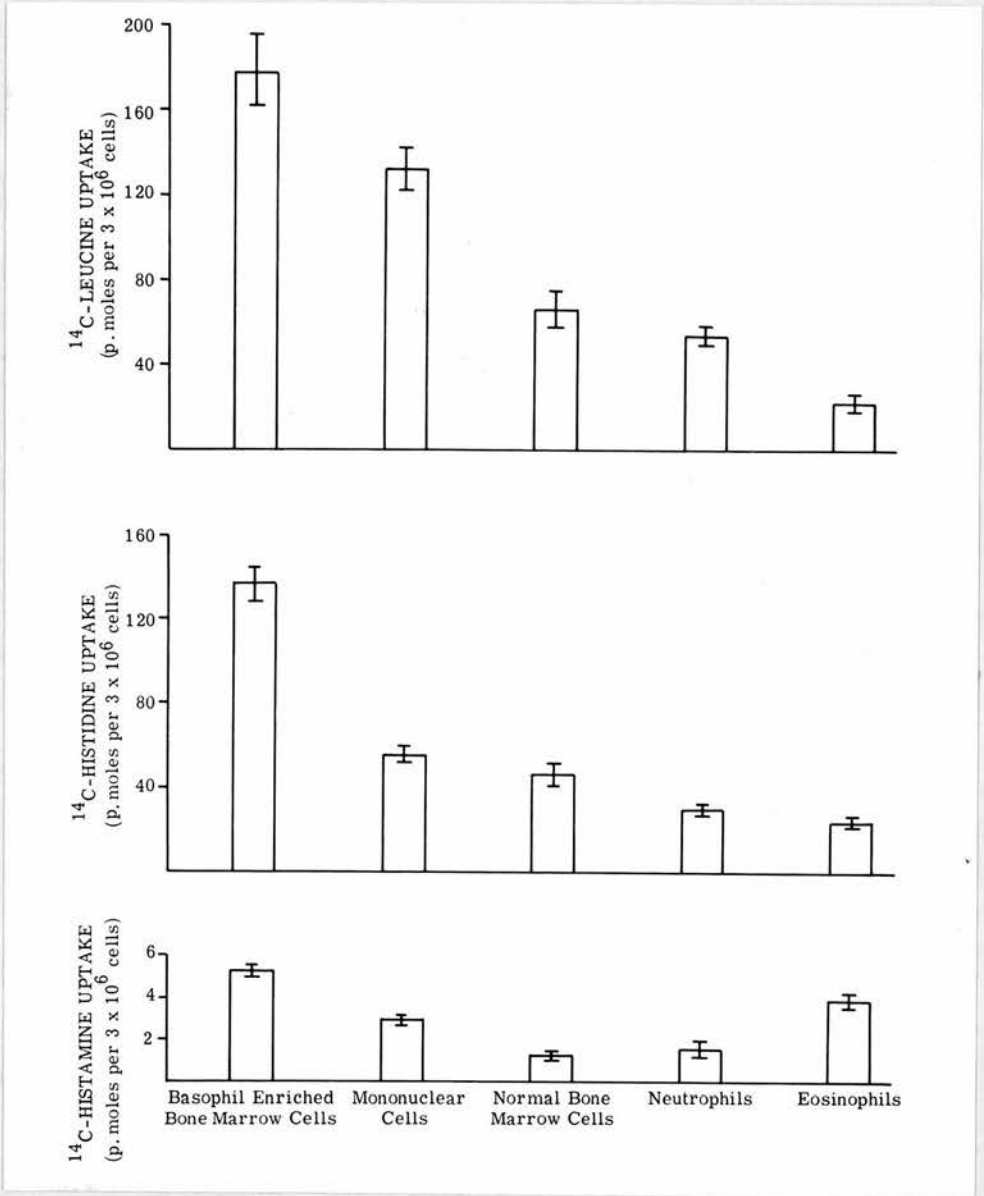

Fig. 8

The uptake of ^{14}C-leucine, ^{14}C-histidine and ^{14}C-histamine by basophil-enriched, mononuclear, normal bone marrow, neutrophil-enriched and eosinophil-enriched cell suspensions. Each column represents the mean ± 1 S.E. ^{14}C-leucine uptake: basophils - 12 experiments; mononuclear cells, neutrophils and eosinophils - 11 experiments; normal bone marrow cells - 15 experiments. ^{14}C-histidine uptake: basophils - 23 experiments; eosinophils and normal bone marrow cells - 16 experiments; neutrophils and mononuclear cells - 14 experiments. ^{14}C-histamine uptake: basophils - 14 experiments; normal bone marrow cells - 7 experiments; neutrophils - 5 experiments; eosinophils and mononuclear cells - 3 experiments.

SOURCE OF BASOPHILS	^{14}C-HISTIDINE UPTAKE (p.moles per 3 x 10^6 cells)	HISTAMINE FORMATION (p.moles per 3 x 10^6 cells)	^{14}C-HISTAMINE UPTAKE (p.moles per 3 x 10^6 cells)
Blood	76.7 ± 20.2	20.0 ± 2.7	6.1 ± 2.2
Bone Marrow	150.4 ± 23.2	36.0 ± 4.7	4.2 ± 0.6

TABLE III

The uptake of ^{14}C-histidine and ^{14}C-histamine and the amount of ^{14}C-histamine formed by cell suspensions rich in basophils derived from blood and bone marrow. The figures represent the mean ± 1 S.E. ^{14}C-histidine uptake and ^{14}C-histamine formation - 8 experiments; ^{14}C-histamine uptake - 3 experiments.

the same there was wide variation between different experiments. Peripheral blood cells incorporated less ^{14}C-histidine and formed a smaller amount of new histamine than bone marrow counterparts although they converted a slightly larger percentage of the ^{14}C-histidine taken up to ^{14}C-histamine. There was no significant difference in the amount of ^{14}C-histamine taken up by basophil preparations from the two sources.

4.3 Kinetic studies on ^{14}C-histidine uptake by different cell types

In part 4.2 it was demonstrated that different cell types varied considerably in their capacity to incorporate ^{14}C-histidine. Experiments were, therefore, designed to determine whether these observations were a reflection of differences in the kinetics of the transport process in these cells. Eosinophil-, neutrophil-, macrophage- and basophil-rich preparations, in addition to normal bone marrow cell suspensions were incubated for 45 min with various concentrations of ^{14}C-histidine (Fig. 9 and 9A). The incubation time was chosen because it appeared from preliminary observations and the data shown in Fig. 2 that the initial velocity was linear during the first 45 min.

Over the concentration range employed some of the cell types appeared to reach a saturated level of uptake. The uptake by eosinophil- and neutrophil-enirched cell suspensions paralleled each other at low concentrations, up to 500 μ.moles.l^{-1}, but tended to drift apart at higher concentrations. At extremely low concentrations, below 0.5 μ.moles.l^{-1}, the normal bone marrow cells followed the same curve as the

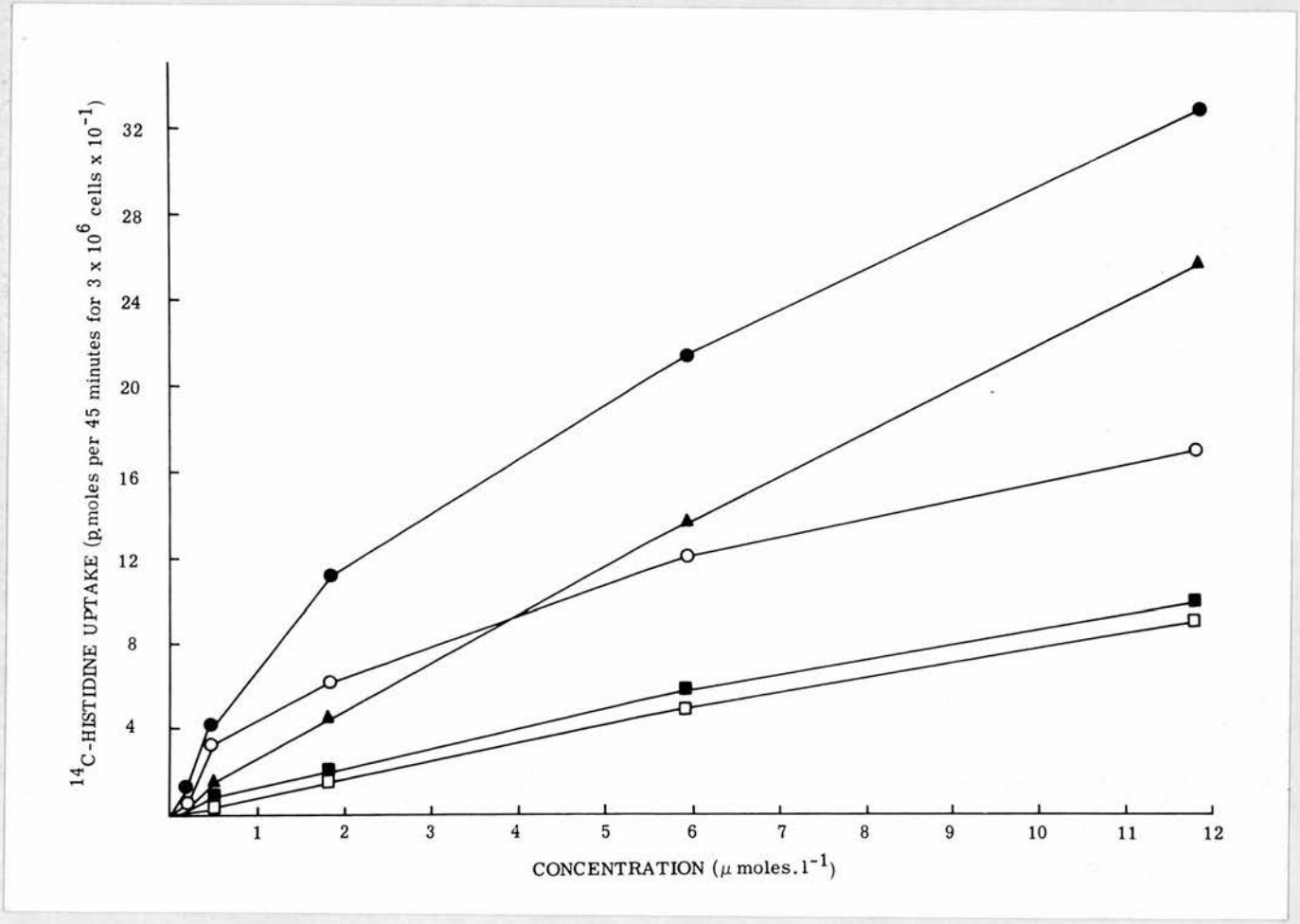

Fig. 9

The effect of increased substrate concentration on the uptake of ^{14}C-histidine by basophil-enriched (● — ●), macrophage-enriched (▲ — ▲), neutrophil-enriched (■ — ■), eosinophil-enriched (□ — □) and normal bone marrow (O — O) cell suspensions. The points represent the mean ± 1 S.E. Basophil-enriched cell suspensions - 6 experiments; macrophage-enriched, neutrophil-enriched, eosinophil-enriched and normal bone marrow cell suspensions - 5 experiments.

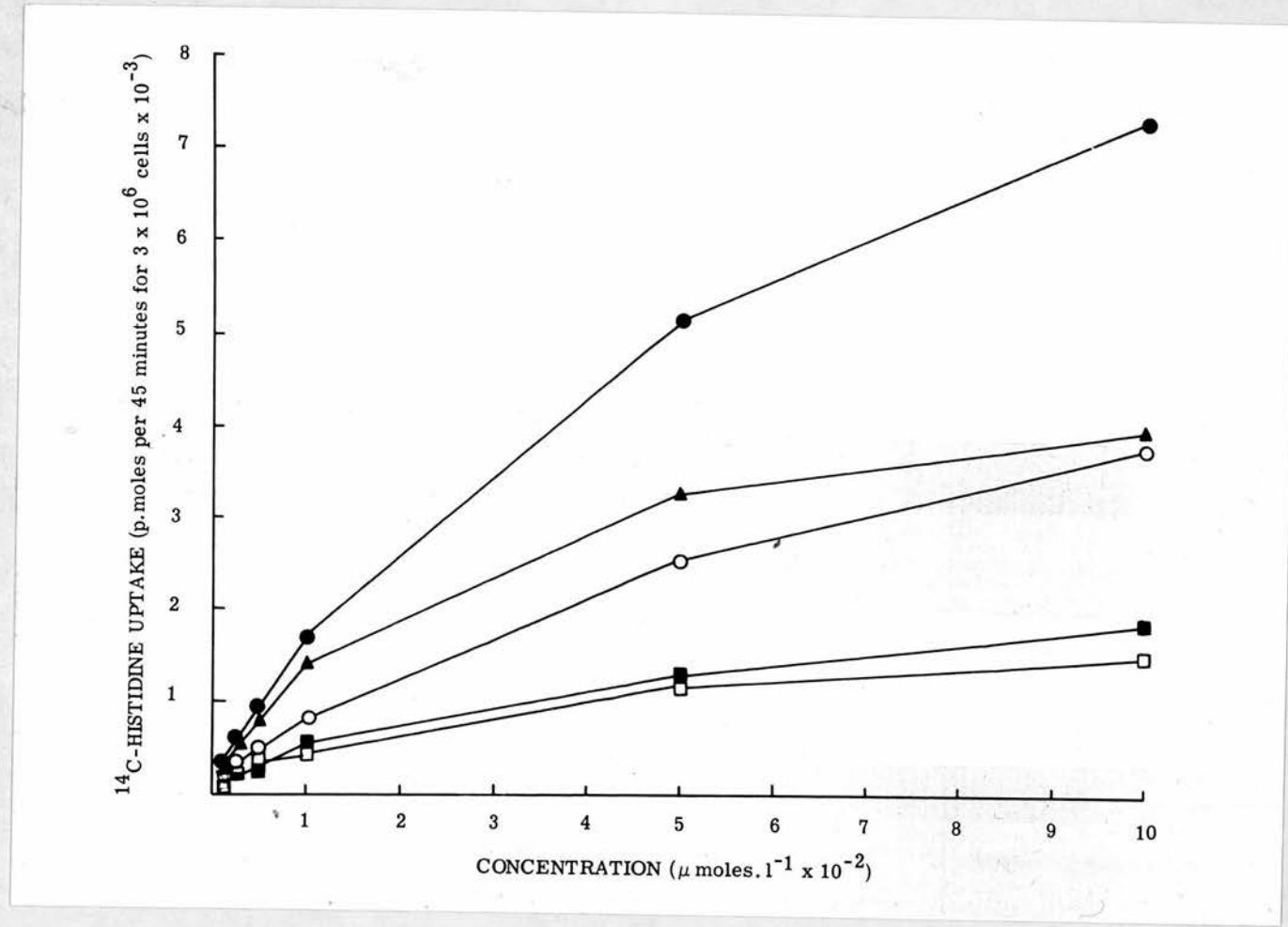

Fig. 9A

The effect of increased substrate concentration on the uptake of ^{14}C-histidine by basophil-enriched (● — ●), macrophage-enriched (▲ — ▲), neutrophil-enriched (■ — ■), eosinophil-enriched (□ — □) and normal bone marrow (O — O) cell suspensions. The points represent the mean ± 1 S.E. Basophil-enriched cell suspensions - 6 experiments; macrophage-enriched, neutrophil-enriched, eosinophil-enriched and normal bone marrow cell suspensions - 5 experiments.

basophil-rich preparations but tailed off more and more as the extracellular histidine concentration was increased. Cell suspensions rich in macrophages had a low uptake rate, at substrate concentrations below 2 μ.moles.l^{-1}, which increased steadily as the concentration of histidine was raised, becoming progressively more similar to that of the basophils before levelling off.

The starting point for analysis of the kinetics of all enzymatic reactions is the Michaelis-Menten relationship. Accordingly the data for the basophil and eosinophil-enriched cell suspensions was fitted to the Michaelis-Menten equation using a computer programme written by Dr. Ian Nimmo. The substrate concentration, experimentally observed velocity, computer derived velocity and residuals (a measure of how well an experimentally derived value agrees with the calculated value) for the basophil and eosinophil-enriched cell suspensions are shown in Table IV. If the uptake of ^{14}C-histidine followed a simple Michaelis-Menten relationship, i.e. a graph of velocity against substrate concentration was a rectangular hyperbole, then an equal number of positive and negative residuals would be anticipated. When the present ^{14}C-histidine uptake data was tested the majority of the residuals were positive, 9 out of 10 and 8 out of 10 for the basophil- and eosinophil-rich preparations respectively.

The data was similarly analysed by another computer programme to determine the Hill coefficients (see Appendix I.2) for all the cell populations used. The calculated velocities and residuals obtained using this programme are given in Tables V and VI. The Hill coefficients (h) were

TABLE IV

The rate of ^{14}C-histidine uptake and the calculated velocities and residuals, obtained by fitting the data to the Michaelis-Menten relationship, for basophil- and eosinophil-enriched cell suspensions incubated with various concentrations of ^{14}C-histidine for 45 min. The experimentally observed and computer derived velocities are expressed as p.moles ^{14}C-histidine incorporated per 45 min for 3 x 10^6 nucleated cells. The experimental results are the mean of 6 experiments for basophil-enriched cell suspensions and 5 experiments for eosinophil-enriched cell suspensions.

	BASOPHILS			EOSINOPHILS		
SUBSTRATE CONCENTRATION (μ moles.l^{-1})	OBSERVED VELOCITY	CALCULATED VELOCITY	RESIDUAL	OBSERVED VELOCITY	CALCULATED VELOCITY	RESIDUAL
0.18	12.5	3.4	9.1	1.4	1.2	0.2
0.45	41.1	8.4	32.7	5.2	2.9	2.3
1.8	111.2	33.7	77.5	16.3	11.7	4.6
5.9	215.8	109.7	106.0	49.7	38.0	11.7
11.8	309.2	217.5	91.7	92.6	74.5	18.1
26.8	562.5	482.7	79.8	194.6	161.3	33.3
51.8	917.3	898.5	18.7	309.5	289.5	20.0
101.8	1677.2	1644.6	32.6	496.2	497.6	-1.4
503.6	5156.4	5243.2	-86.7	1179.0	1226.0	-47.4
1005.4	7291.8	7248.8	43.0	1535.7	1505.2	30.0

TABLE IV

TABLE V

The rate of ^{14}C-histidine uptake and the calculated velocities and residuals, obtained by fitting the data to the Hill equation, for basophil- and eosinophil-enriched cell suspensions as in Table IV.

	BASOPHILS			EOSINOPHILS		
SUBSTRATE CONCENTRATION (μ moles.l^{-1})	OBSERVED VELOCITY	CALCULATED VELOCITY	RESIDUAL	OBSERVED VELOCITY	CALCULATED VELOCITY	RESIDUAL
0.18	12.5	6.5	6.0	1.4	3.4	-2.0
0.45	41.1	14.9	26.2	5.2	7.2	-2.0
1.8	111.2	51.3	59.9	16.3	22.2	-5.9
5.9	215.8	147.6	68.3	49.7	57.9	-8.2
11.8	309.2	272.1	37.1	92.6	100.4	-7.8
26.8	562.5	555.7	67.5	194.6	189.3	5.3
51.8	917.3	972.4	-55.1	309.5	308.0	1.5
101.8	1677.2	1685.9	-8.7	496.2	490.7	5.5
503.6	5156.4	5135.9	20.5	1179.0	1185.5	-6.4
1005.4	7291.8	7300.6	-8.8	1535.7	1532.5	3.2

TABLE V

TABLE VI

The rate of ^{14}C-histidine uptake and the calculated velocities and residuals, obtained by fitting the data to the Hill equation, for normal bone marrow, mononuclear and neutrophil-enriched cell suspensions as in Table IV.

SUBSTRATE CONCENTRATION (μ moles.l^{-1})	NORMAL BONE MARROW CELLS			MONONUCLEAR CELLS			NEUTROPHILS		
	OBSERVED VELOCITY	CAL-CULATED VELOCITY	RESIDUAL	OBSERVED VELOCITY	CAL-CULATED VELOCITY	RESIDUAL	OBSERVED VELOCITY	CAL-CULATED VELOCITY	RESIDUAL
0.18	4.0	4.5	-0.5	3.4	4.4	-0.9	1.7	4.6	-2.9
0.45	32.7	9.5	23.2	15.6	10.5	5.1	7.7	9.2	-1.5
1.8	61.0	30.1	30.9	45.8	39.4	6.4	19.3	25.8	-6.6
5.9	118.6	80.3	38.3	135.5	120.9	14.5	57.4	62.6	-5.2
11.8	173.9	142.0	31.9	257.6	229.8	27.8	99.0	104.4	-5.4
26.8	269.7	277.2	-7.5	524.5	478.5	46.0	174.8	189.7	-14.9
51.8	454.6	470.6	-16.0	779.9	831.1	-51.2	296.6	303.4	-6.8
101.8	778.5	799.2	-20.7	1370.0	1383.0	-13.0	514.4	483.5	30.9
503.6	2526.2	2510.8	15.4	3260.5	3229.5	31.0	1291.4	1306.4	-15.0
1005.4	3783.4	3789.0	-5.6	3913.0	3930.8	-17.8	1847.1	1841.3	5.8

TABLE VI

all less than one (Table VII) suggesting that negative co-operativity was occurring. The calculated maximum velocity (V_{MAX}) and the substrate concentrations which gave half V_{MAX} (s_{50}) are also given for each cell population in Table VII.

The present results suggest that ^{14}C-histidine uptake does not follow simple Michaelis-Menten kinetics. At low substrate concentrations the uptake was greater than would be predicted by the Michaelis-Menten relationship and at high concentrations it was lower than predicted, i.e. negative co-operativity (see Appendix I.1 for possible explanation).

The calculated velocities and residuals for a simulation, containing two K_M values, obtained by fitting the data calculated in Appendix I.3 to the Hill equation, are given in Table VIII. The Hill coefficient (Table VII) was negative, therefore a system containing more than one transport system with different K_M's would probably exhibit negative co-operativity.

4.4 Influence of various amino acids on ^{14}C-histidine uptake and histamine formation

The addition of unlabelled L-histidine had a dramatic effect on the uptake of ^{14}C-histidine and ^{14}C-histamine formation, whereas D-histidine had no effect on ^{14}C-histidine uptake, although it appeared to decrease slightly ^{14}C-histamine formation (Fig. 10).

At all concentrations of L-histidine used there was a significant decrease ($p < 0.001$) in the amount of ^{14}C-histidine incorporated and ^{14}C-histamine synthesized. ^{14}C-histidine uptake was unaffected by D-histidine but at the highest concentration used, 10^{-3} moles.l^{-1}, there was a slight

CELL TYPE	V_{MAX}	K	h	s_{50}
Basophils	14361.2	472.1	0.90	935.6
Normal Bone Marrow	11015.5	595.1	0.83	2203.0
Mononuclear	5113.0	226.0	0.96	283.1
Neutrophils	4758.0	284.3	0.78	1869.0
Eosinophils	2489.3	179.5	0.82	561.0
Simulation	1.99	1.36	0.95	1.38

TABLE VII

The maximum veolcity (V_{MAX}), Michaelis-Menten constant (K), Hill constant (h) and substrate concentration which gives half V_{MAX} (s_{50}) for basophil-enriched, normal bone marrow, mononuclear, neutrophil-enriched and eosinophil-enriched cell suspensions and a simulation. For the various cell types V_{MAX} is expressed as p.moles ^{14}C-histidine incorporated per 45 min for 3×10^6 nucleated cells and s_{50} has units of μ.moles.l^{-1}. The values for the simulation are arbitrary.

SUBSTRATE CONCENTRATION (units)	OBSERVED VELOCITY (units)	CALCULATED VELOCITY (units)	RESIDUAL
0.5	0.5333	0.5509	-0.018
0.75	0.7504	0.7162	0.034
1.0	0.8333	0.8453	-0.012
1.5	1.0286	1.0354	-0.007
2.0	1.1667	1.1693	-0.003
2.5	1.2698	1.2691	0.001
3.0	1.35	1.3466	0.003

TABLE VIII

The computer derived velocities and residuals, obtained by fitting the data to the Hill equation, for a simulated experiment where the substrate concentration and observed velocities were calculated from the Michaelis-Menten relationship.

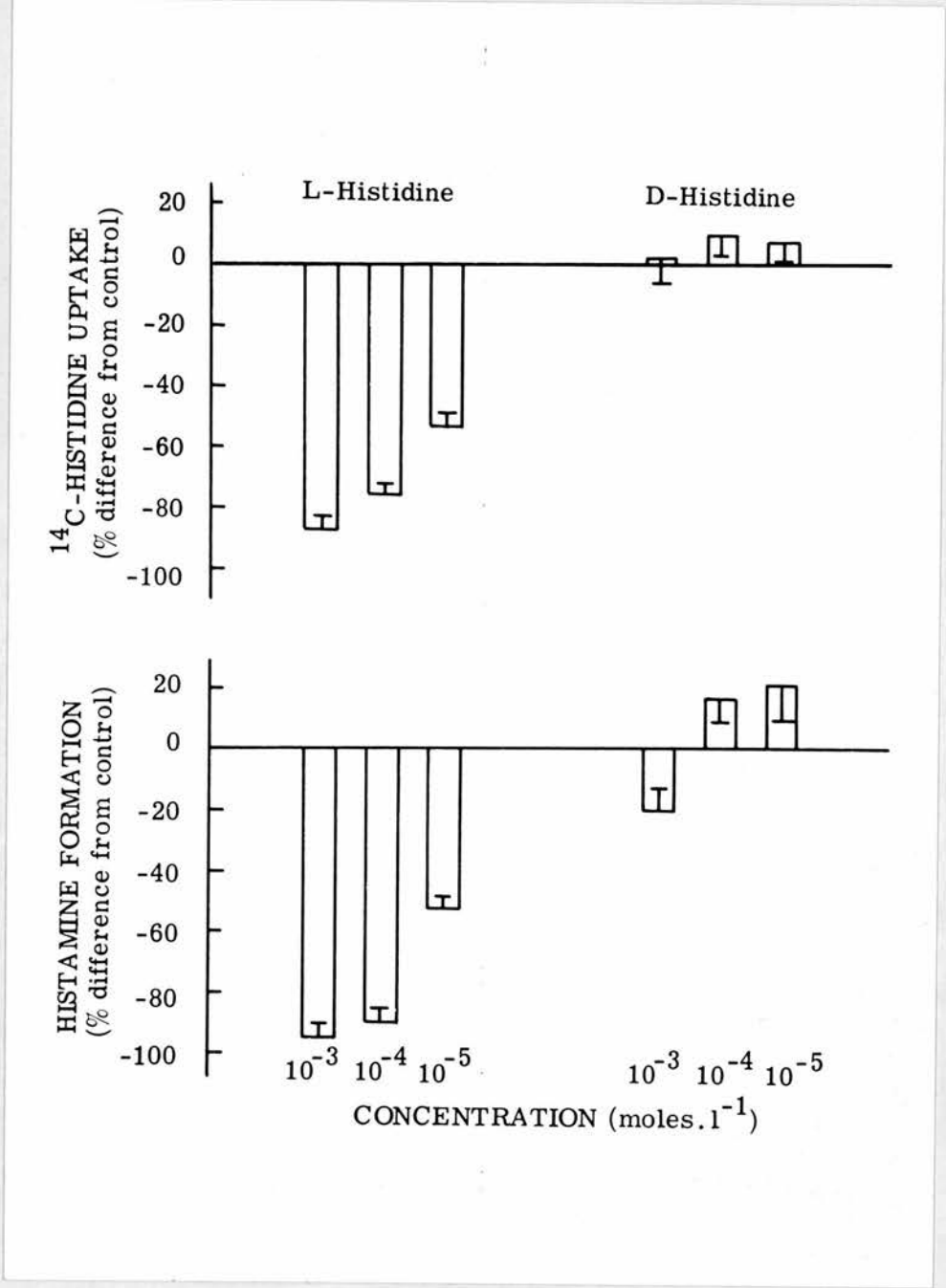

Fig. 10

The effect of L- and D-histidine on the uptake of ^{14}C-histidine and ^{14}C-histamine formation. Each point represents the mean ± 1 S.E. of 6 experiments.

but statistically significant ($p < 0.05$) inhibition of ^{14}C-histamine formation.

The effect of most naturally occurring amino acids on ^{14}C-histidine uptake and histamine formation is shown in Figs. 11 to 15.

Tryptophan gave significant inhibition of ^{14}C-histidine uptake at all concentrations used, 10^{-3} moles.l^{-1} ($p < 0.01$) and 10^{-4} and 10^{-5} moles.l^{-1} ($p < 0.05$). Its inhibition of ^{14}C-histamine synthesis was significant at 10^{-3} moles.l^{-1} ($p < 0.001$) and 10^{-4} moles.l^{-1} ($p < 0.05$). Tyrosine produced significant inhibition of ^{14}C-histidine uptake at 10^{-3} moles.l^{-1} ($p < 0.01$) and 10^{-4} moles.l^{-1} ($p < 0.05$). At all concentrations it significantly decreased ^{14}C-histamine synthesis.

The sulphur containing amino acids, methionine and cysteine, produced dose-dependent inhibition of ^{14}C-histidine uptake and ^{14}C-histamine synthesis. Methionine significantly decreased ^{14}C-histidine uptake and ^{14}C-histamine synthesis at 10^{-3} moles.l^{-1} ($p < 0.01$ and $p < 0.001$ respectively), the latter also being significantly inhibited at 10^{-4} moles.l^{-1} ($p < 0.05$). At 10^{-3} ($p < 0.01$) and 10^{-4} moles.l^{-1} ($p < 0.05$) cysteine decreased the amount of ^{14}C-histidine incorporated, at the same concentrations it also inhibited ^{14}C-histamine formation ($p < 0.01$).

The three hydrophobic amino acids, phenylalanine, leucine and isoleucine, inhibited ^{14}C-histidine uptake and ^{14}C-histamine formation in a dose-dependent fashion (Fig. 12). Phenylalanine and isoleucine significantly decreased ^{14}C-histidine uptake and ^{14}C-histamine formation at 10^{-3} moles.l^{-1} ($p < 0.001$), 10^{-4} moles.l^{-1} ($p < 0.01$) and 10^{-5} moles.l^{-1} ($p < 0.05$). The inhibition of ^{14}C-histidine uptake by leucine

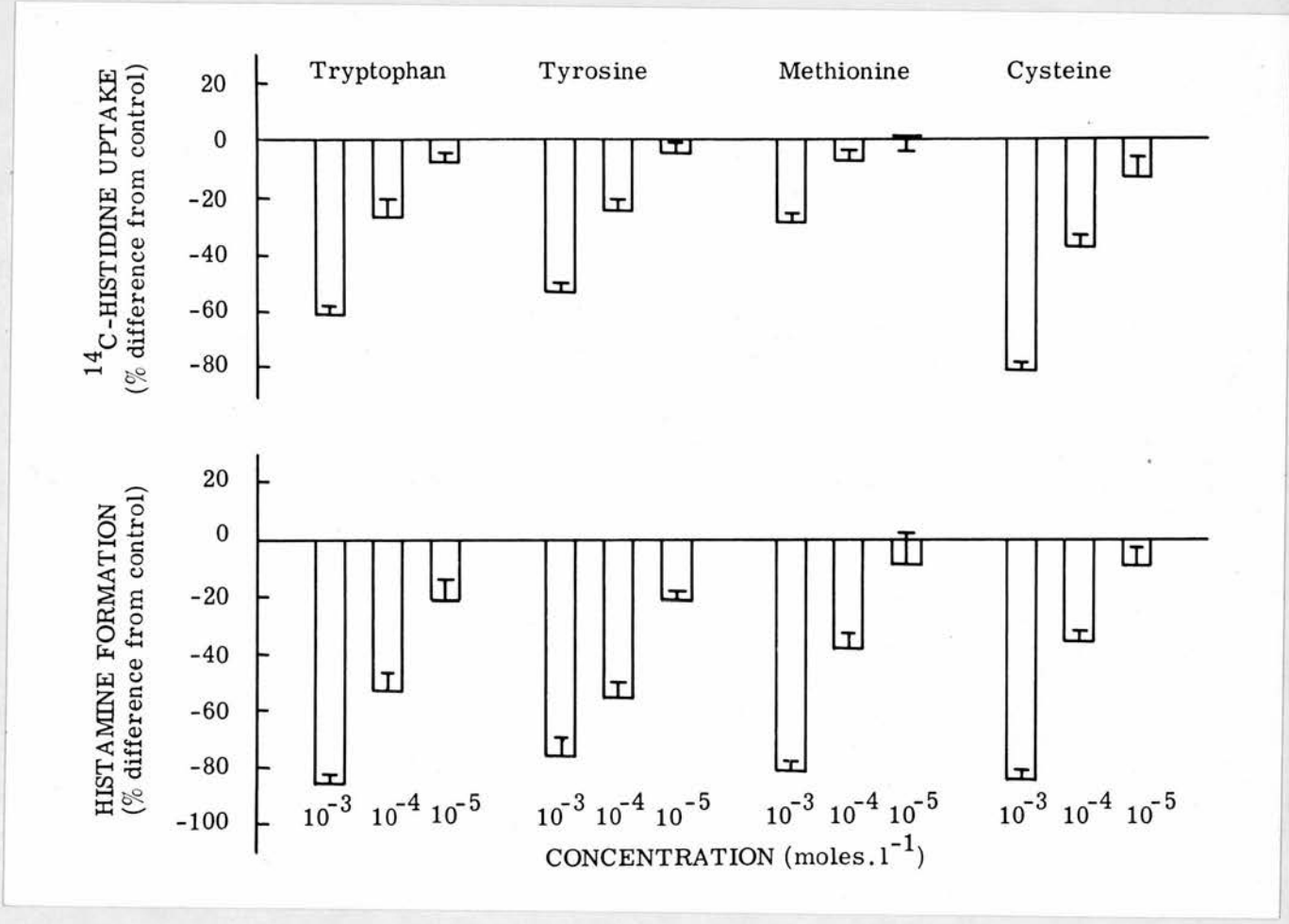

Fig. 11

The effect of amino acids on the uptake of ^{14}C-histidine and ^{14}C-histamine formation. Each column represents the mean ± 1 S.E. Tryptophan, tyrosine and cysteine - 3 experiments; methionine - 4 experiments.

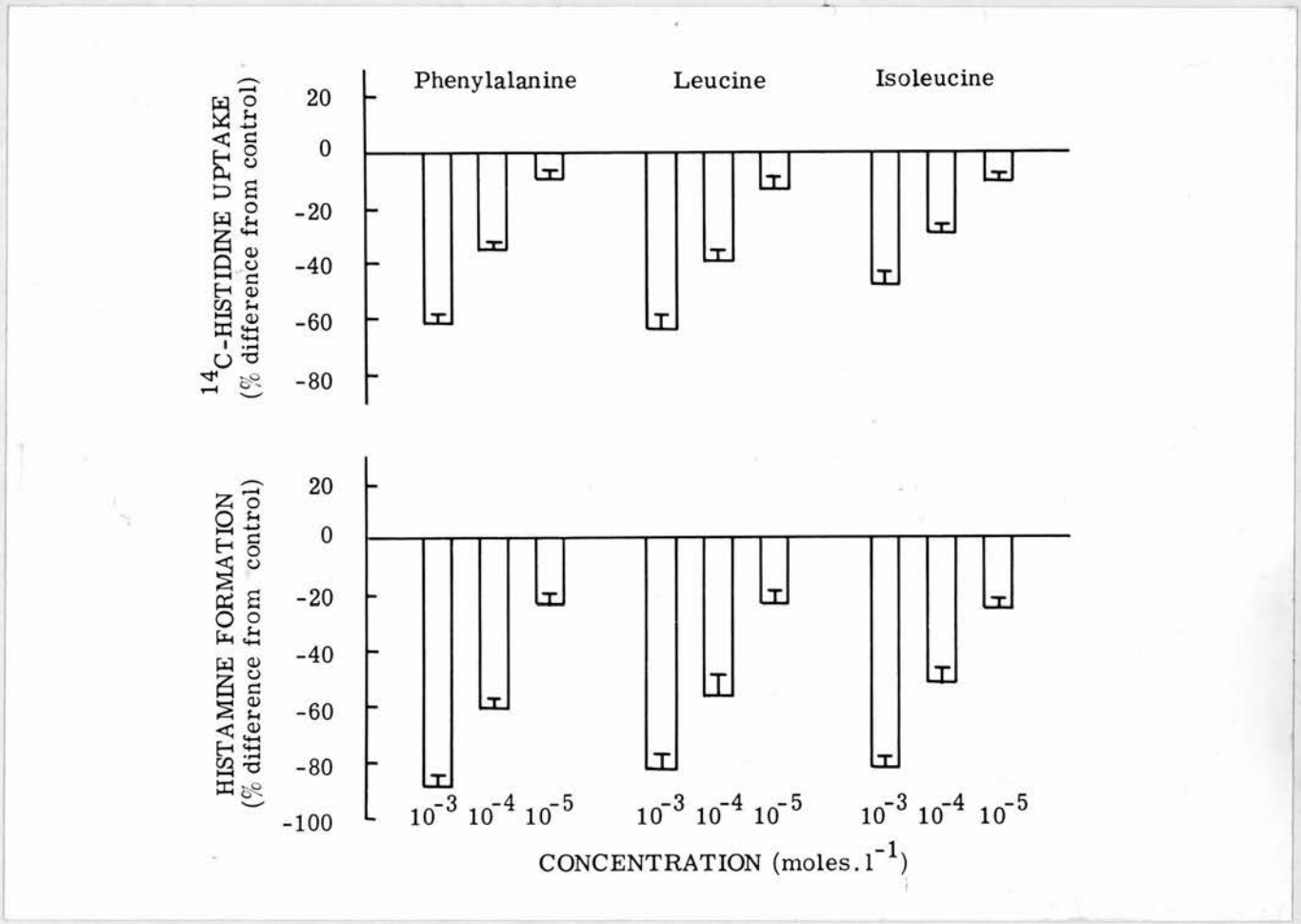

Fig. 12

The effect of amino acids on the uptake of ^{14}C-histidine and ^{14}C-histamine formation. Each column represents the mean ± 1 S.E. Phenylalanine, and leucine - 3 experiments; isoleucine - 4 experiments.

was significant at 10^{-3} moles.l^{-1} ($p < 0.01$) and 10^{-4} moles.l^{-1} ($p < 0.001$). Its inhibitory effect on ^{14}C-histamine formation was significant at all concentrations used, 10^{-3} moles.l^{-1} ($p < 0.01$) and 10^{-4} and 10^{-5} moles.l^{-1} ($p < 0.05$).

Alanine, serine and threonine had no statistically significant effect on ^{14}C-histidine uptake but at high concentrations decreased the amount of ^{14}C-histamine synthesized (Fig. 13). Alanine and serine at 10^{-3} moles.l^{-1} and threonine at 10^{-3} and 10^{-4} moles.l^{-1} significantly inhibited ^{14}C-histamine formation ($p < 0.05$).

The effect of valine, lysine and glutamine on ^{14}C-histidine uptake and ^{14}C-histamine formation is shown in Fig. 14. Valine and lysine at 10^{-3} moles.l^{-1} significantly inhibited ($p < 0.01$) ^{14}C-histidine uptake whereas at all concentrations used glutamine was without effect. ^{14}C-histamine synthesis was significantly decreased by valine at 10^{-3} moles.l^{-1} ($p < 0.001$) and 10^{-4} moles.l^{-1} ($p < 0.01$), and by lysine and glutamine at 10^{-3} moles.l^{-1} ($p < 0.05$).

Proline, glycine, cystine, arginine, aspartic acid, asparagine and glutamic acid had no significant effect on either ^{14}C-histidine uptake or ^{14}C-histamine formation over the concentration range studied (Fig. 15).

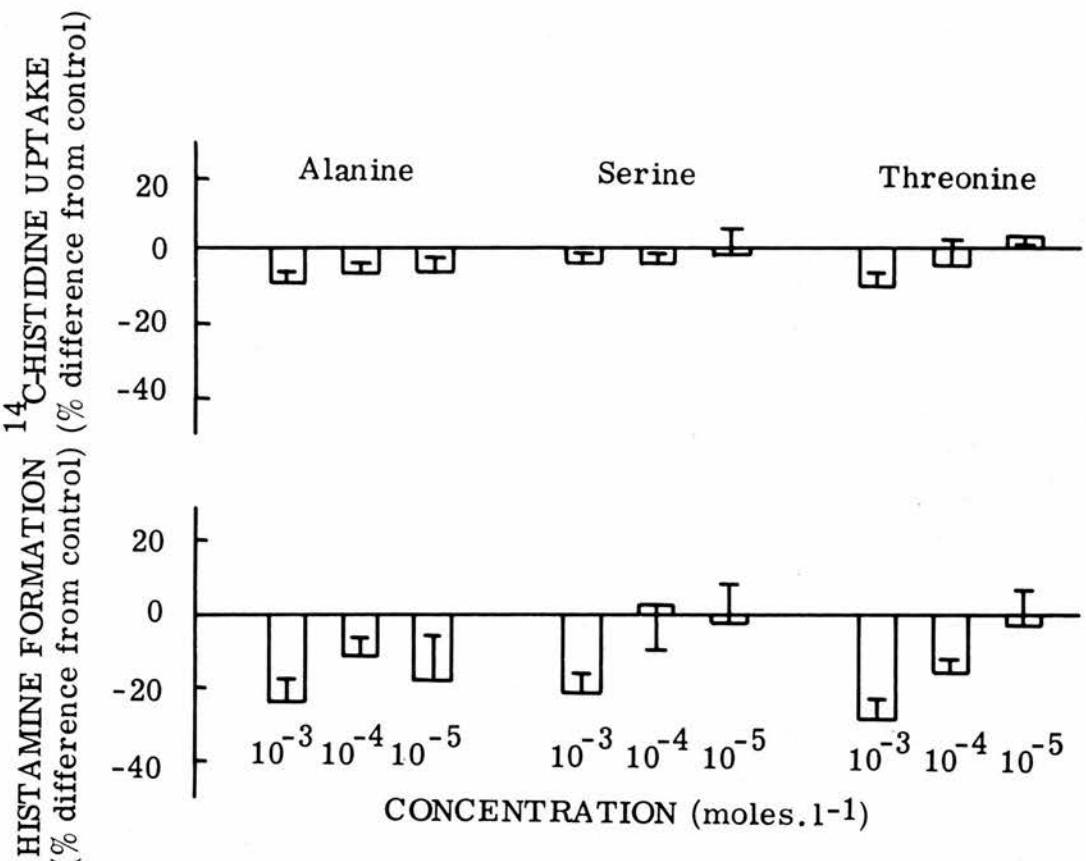

Fig. 13

The effect of amino acids on the uptake of ^{14}C-histidine and ^{14}C-histamine formation. Each column represents the mean ± 1 S.E. Alanine, serine and threonine - 4 experiments.

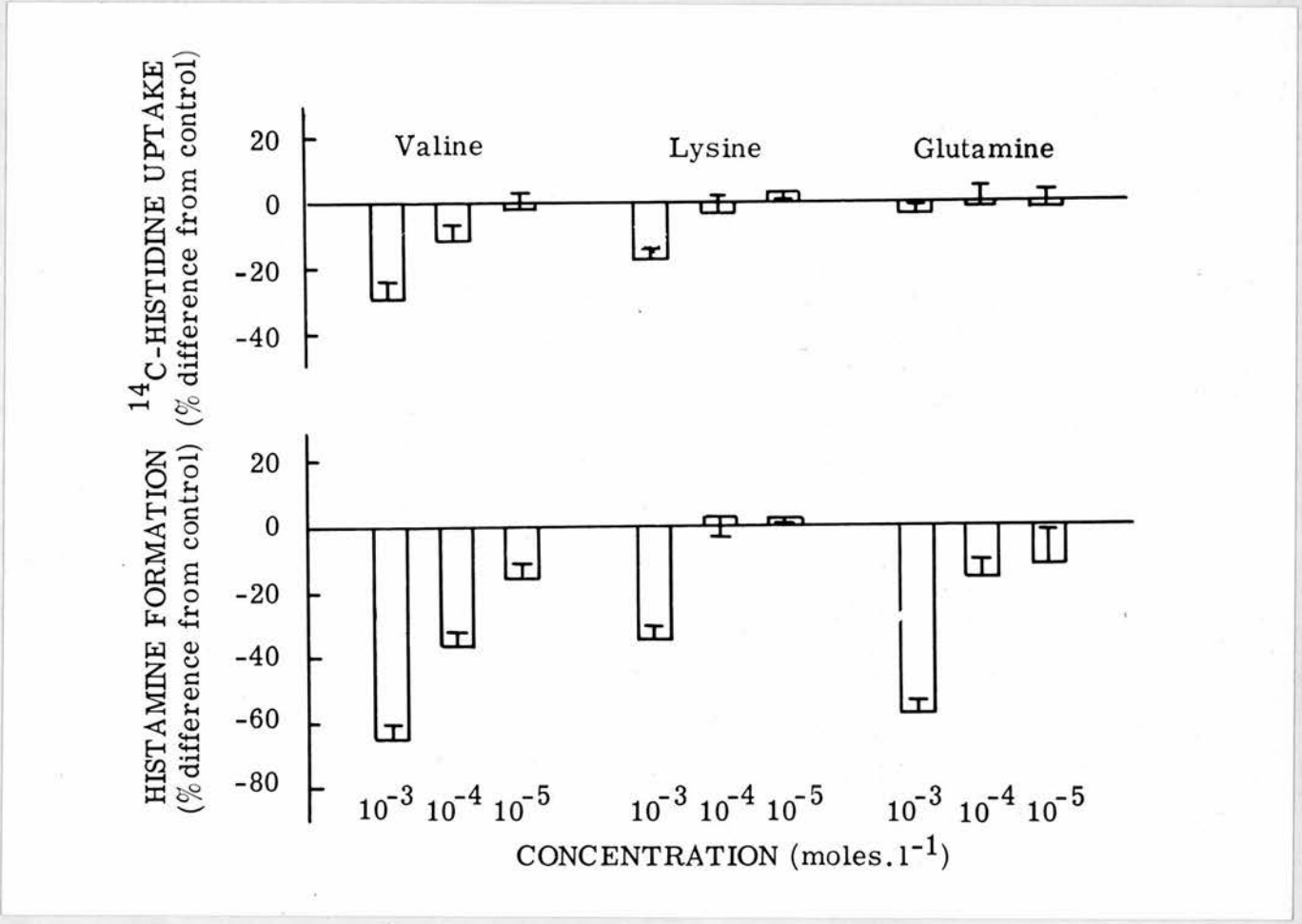

Fig. 14

The effect of amino acids on the uptake of ^{14}C-histidine and ^{14}C-histamine formation. Each column represents the mean ± 1 S.E. Valine, lysine and glutamine - 4 experiments.

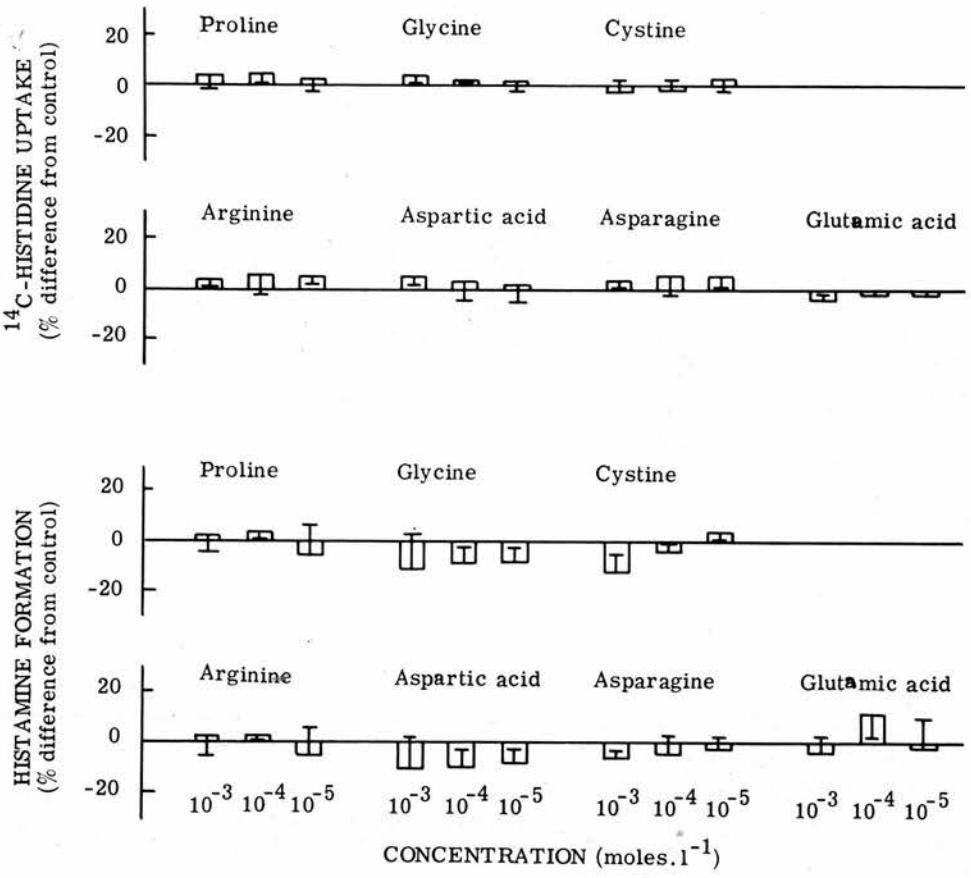

Fig. 15

The effect of amino acids on the uptake of ^{14}C-histidine and ^{14}C-histamine formation. Each column represents the mean ± 1 S.E. Proline, glycine, arginine, aspartic acid and glutamic acid - 4 experiments; cysteine and asparagine - 3 experiments.

5.0 COMPARISON WITH RAT MAST CELLS

The uptake of ^{14}C-histidine and ^{14}C-histamine and ^{14}C-histamine formation was studied in purified preparations of rat peritoneal mast cells. The amounts of ^{14}C-histidine and ^{14}C-histamine incorporated, ^{14}C-histamine formed de novo and endogenous histamine are shown in Table IX along with similar data for the guinea pig bone marrow basophil.

The values reported are for pure mast cell and basophil populations. Since mast cells and basophils were never obtained as pure preparations the uptake of ^{14}C-histidine and ^{14}C-histamine was calculated by subtracting the amounts incorporated by the contaminating cells. This was possible since the contaminating cells could be obtained free of mast cells or basophils.

The guinea pig basophil incorporated about four times as much ^{14}C-histidine as the rat mast cell on a cell to cell basis under the same conditions. However, the rat mast cell converted approximately 83% of the incorporated ^{14}C-histidine to ^{14}C-histamine whereas the basophil converted only 31%. There was no significant difference in ^{14}C-histamine uptake between the two cell types. The amount of ^{14}C-histamine taken up was extremely low compared to the amount formed from the ^{14}C-histidine incorporated. Rat peritoneal mast cells contained approximately ten times more histamine than guinea pig basophils.

	RAT MAST CELLS	GUINEA PIG BASOPHILS
^{14}C-histidine uptake (p moles per 10^6 cells)	33.3 ± 4.7	121.9 ± 12.3
Histamine formation (p moles per 10^6 cells)	27.7 ± 5.7	37.9 ± 3.5
^{14}C-histamine uptake (p moles per 10^6 cells)	5.2 ± 1.1	5.9 ± 0.7
Histamine content (μg per 10^6 cells)	12.5 ± 1.8	1.13 ± 0.08

TABLE IX

The uptake of ^{14}C-histidine and ^{14}C-histamine, amount of ^{14}C-histamine formed and endogenous histamine content of rat peritoneal mast cells and guinea pig bone marrow basophils. The figures represent the mean ± 1 S.E. Rat mast cells: ^{14}C-histidine uptake - 14 experiments; ^{14}C-histamine formation - 8 experiments; ^{14}C-histamine uptake - 4 experiments; histamine content - 6 experiments. Guinea pig basophils: ^{14}C-histidine and ^{14}C-histamine uptake and ^{14}C-histamine formation - 17 experiments; histamine content - 8 experiments.

6.0 SUMMARY

The uptake of ^{14}C-histidine and ^{14}C-histamine and the conversion of the incorporated ^{14}C-histidine to ^{14}C-histamine was measured in suspensions of guinea pig bone marrow cells rich in basophils.

When the same amounts of ^{14}C-labelled histidine and histamine were added to equal numbers of basophils the uptake of histidine was approximately 35 times greater than that of histamine.

Highly purified preparations of eosinophils, neutrophils and mononuclear cells and normal bone marrow cells incorporated less than half as much ^{14}C-histidine when compared to basophil-enriched cell suspensions; ^{14}C-histamine uptake by all cell types was virtually negligible.

^{14}C-histidine and ^{14}C-histamine uptake and ^{14}C-histamine formation was dependent on the time of incubation, substrate concentration and temperature but only ^{14}C-histamine uptake varied significantly with pH.

Kinetic studies showed that the uptake of histidine, under the conditions used, did not fit a simple Michaelis-Menten relationship. However, s_{50} and V_{MAX} values for the various cell preparations were determined.

Thirteen out of the twenty amino acids tested gave some degree of inhibition of ^{14}C-histidine uptake and/or ^{14}C-histamine formation. The pattern of inhibition obtained fitted that which would be expected from transport studies on other mammalian cell types.

APPENDIX TO SECTION I

APPENDIX I.1. POSSIBLE REASONS FOR THE OBSERVATION OF NEGATIVE CO-OPERATIVITY IN THE UPTAKE OF ^{14}C-HISTIDINE

Negative co-operativity would occur if the measured velocity was not linear, i.e. an initial velocity was not being measured. It may be that at high substrate concentrations the rate of uptake was not linear for the entire 45 min incubation and a shorter time should have been used.

Another possibility is that more than one Michaelis-Menten constant (K_M) was present in each set of data since none of the cell preparations was completely homogeneous, either in terms of cell type or maturity. To investigate this theory data containing two K_M's was composed (Appendix I.3) and the Hill equation fitted by computer.

Thirdly, there is no reason why the results obtained could not be due to real effects. It is possible that the transport systems studied do not behave like enzymes and the uptake of histidine follows different relationships.

APPENDIX I.2. DEFINITION OF HILL EQUATION

The Hill equation was first proposed to explain the reaction of oxygen with haemoglobin (Barcroft and Hill, 1909). However, it can also be applied to enzyme catalysed reactions in which case it simplifies to:

$$v = \frac{V.\ s^h}{K + s^h}$$

where V = maximum velocity
s = substrate concentration
K = Michaelis-Menten constant
v = velocity
h = Hill coefficient

If $v = \frac{1}{2}V$ then,

$$\frac{1}{2}V = \frac{V.s^h}{K + s^h}$$

$$K + s^h = 2s^h$$

$$K = s^h$$

Taking logarithms of each side,

$$\log K = h \log s$$

$$\log s = {}^{1}/_{h} \log K = \log K^{\,1/h}$$

. . substrate concentration to give $\frac{1}{2}$ V $(s_{50}) = K^{\,1/h}$

A Hill coefficient of 1 infers that a plot of velocity against substrate concentration is hyperbolic - simple Michaelis-Menten relationship. If the value is greater than 1 then a positive co-operative effect is occurring and if it is less than 1 then negative co-operativity is present.

APPENDIX I.3. CALCULATION OF SIMULATION DATA

The Michaelis-Menten equation:

$$v = \frac{V.\ s}{K + s}$$

where v = velocity

V = maximum velocity

s = substrate concentration

K = Michaelis-Menten constant

defines the quantitative relationship between enzyme reaction rate and substrate concentration if both K and V are known.

To compile the simulation data V was taken to be 1 and the K values 1 and 2.

$$\therefore \quad v_1 = \frac{s}{1 + s} \qquad \text{and} \qquad v_2 = \frac{s}{2 + s}$$

hence the observed velocity $(v_{obs}) = \frac{s}{1 + s} + \frac{s}{2 + s}$

A set of observed velocities was calculated for seven substrate concentrations between 0.5 and 3.0. These values along with the computer derived velocities and residuals are given in Table VIII.

SECTION II - HISTIDINE AND HISTAMINE UPTAKE AND NEW HISTAMINE FORMATION BY BASOPHILS EFFECT OF METABOLIC INHIBITORS

1.0 INTRODUCTION

From the results in Section I the basophil appears to have the capacity to preferentially take up exogenous histidine, as compared to other cell types, and (as also shown by Galli *et al* (1976)) to convert this incorporated histidine to histamine. The uptake of histidine, which appears to be the main source of histamine, fulfils the three characteristics of mediated transport, i.e. substrate specificity, saturatability and specific inhibition. Therefore, further experiments were carried out to determine if the uptake occurred by an active or passive mediated process.

The most fundamental criterion of an active mediated process is transport against a chemical or electrochemical gradient. This requires precise knowledge of the concentration of the transported substance in the two compartments concerned. However, it is not always possible to establish from analytical measurements, if these can be made, whether or not a true thermodynamic gradient exists.

A second criterion of active mediated transport, but not necessarily an infallible test, is to determine if the process is dependent upon a source of metabolic energy. Therefore, the influence of a number of metabolic inhibitors on histidine uptake was investigated. Their effects on histamine uptake were examined since it was unclear from Section I if this process was mediated or not. Histamine formation *de novo* was also studied in the presence of the various metabolic inhibitors to determine if inhibition of histidine uptake led to a decrease in the amount of histamine formed.

2.0 EFFECT OF METABOLIC INHIBITORS ON ^{14}C-HISTIDINE UPTAKE

The effect of various metabolic inhibitors on the uptake of ^{14}C-histidine is shown in Figs. 16-18. The doses chosen were those which gave the same degree of cell viability as diluent controls.

2.1 General metabolic inhibitors

Iodoacetamide decreased the amount of ^{14}C-histidine incorporated in a dose-dependent fashion producing a significant inhibition at 10^{-4} moles.l^{-1} ($p < 0.01$). NSD 1055, an inhibitor of L-aromatic amino acid decarboxylase and specific histidine decarboxylase, gave significant inhibition at all the concentrations used, at 10^{-4} and 10^{-6} moles.l^{-1} ($p < 0.001$) and 10^{-5} moles.l^{-1} ($p < 0.05$).

2.2 Inhibitors of microtubule and microfilament function

Colchicine had no effect on ^{14}C-histidine uptake over the concentration range 10^{-4} to 10^{-6} moles.l^{-1}. Cytochalasin B gave significant inhibition at 10^{-6} moles.l^{-1} ($p < 0.001$).

2.3 Inhibitors of glycolysis and agents which influence aerobic respiration

The inhibitors of glycolysis, 2-deoxy-D-glucose and sodium fluoride, decreased the amount of ^{14}C-histidine taken up by the basophil-rich cell suspensions (Fig. 17). 2-deoxy-D-glucose produced significant inhibition at 10^{-3} moles.l^{-1} ($p < 0.01$) and 10^{-4} moles.l^{-1} ($p < 0.05$). At 10^{-2} and 10^{-3} moles.l^{-1} sodium fluoride significantly ($p < 0.01$) decreased the uptake of ^{14}C-histidine.

2,4-dinitrophenol, an uncoupler of oxidative phosphorylation, and antimycin A, a respiratory chain inhibitor,

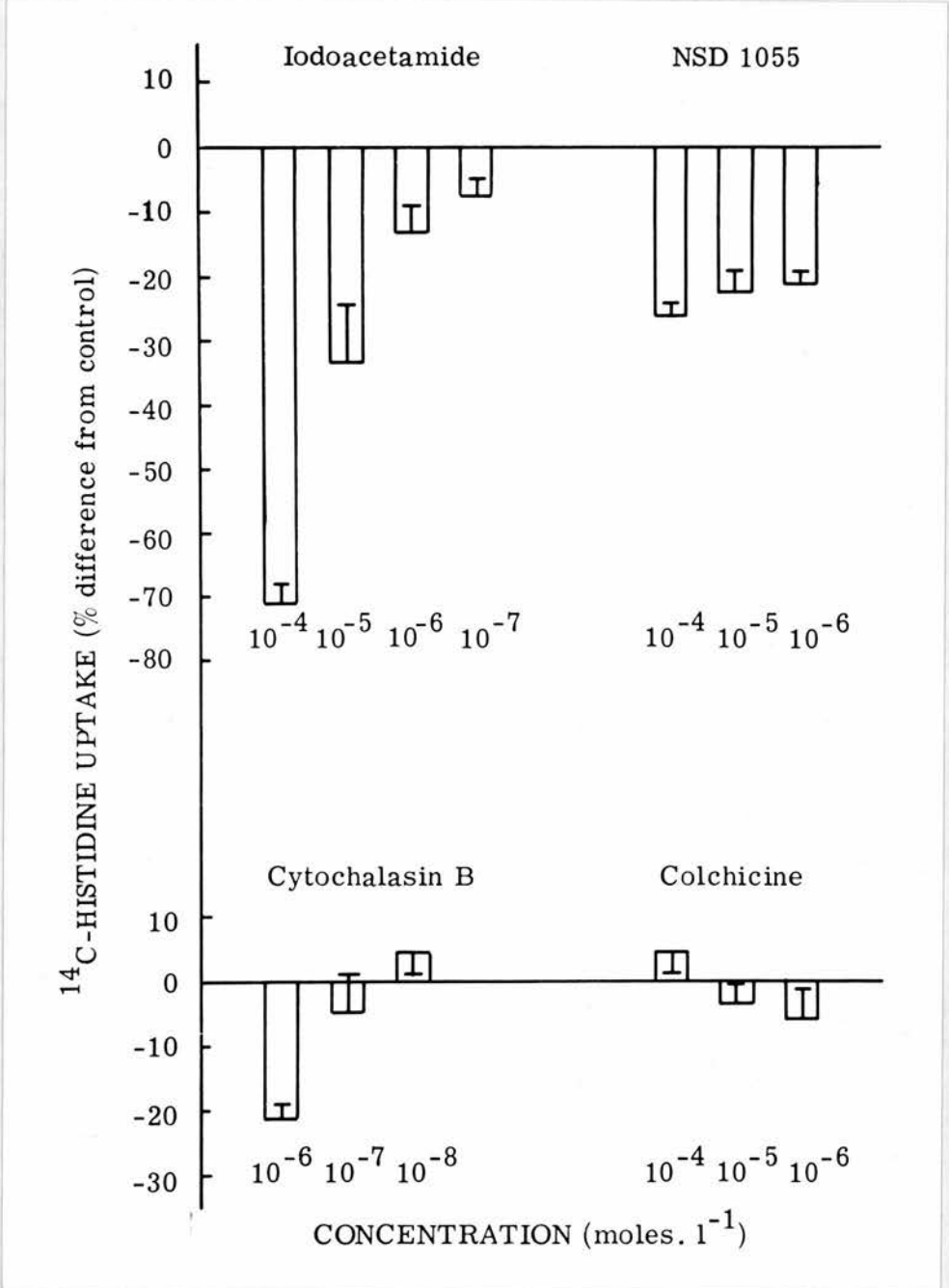

Fig. 16

The effect of metabolic inhibitors on the uptake of ^{14}C-histidine. Each column represents the mean ± 1 S.E. Colchicine and cytochalasin B (10^{-6} mol.l^{-1}) - 6 experiments; cytochalasin B (10^{-7} mol.l^{-1}) - 5 experiments; NSD 1055 and cytochalasin B (10^{-8} mol.l^{-1}) - 4 experiments; iodoacetamide - 3 experiments.

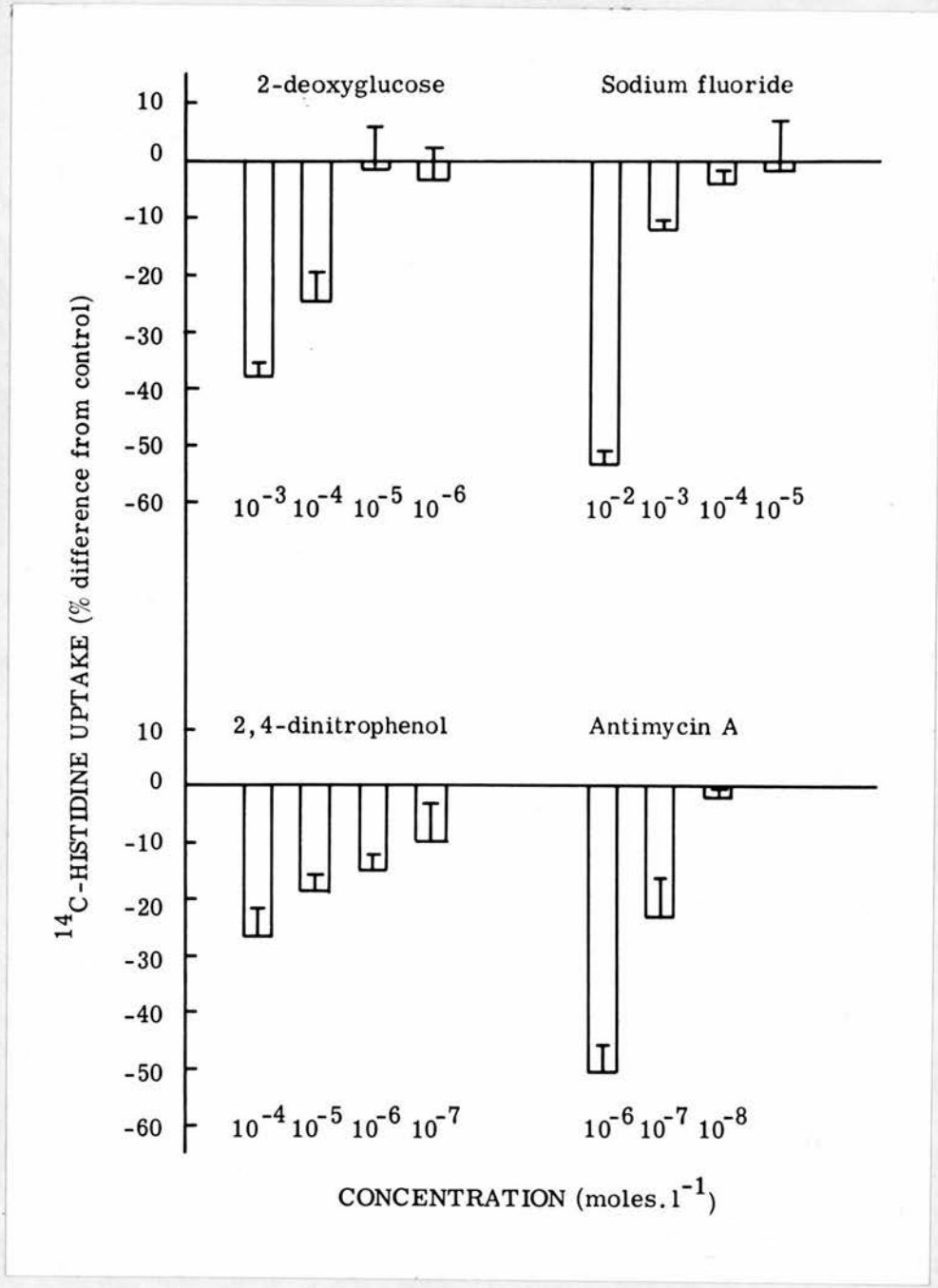

Fig. 17

The effect of metabolic inhibitors on the uptake of ^{14}C-histidine. Each column represents the mean ± 1 S.E. Antimycin A (10^{-6} mol.l^{-1}) - 6 experiments; sodium fluoride (10^{-3} and 10^{-4} mol.l^{-1}), 2,4-dinitrophenol (10^{-5} and 10^{-6} mol.l^{-1}) and antimycin A (10^{-7} mol.l^{-1}) - 5 experiments; 2,4-dinitrophenol (10^{-4} mol.l^{-1}) and antimycin A (10^{-8} mol.l^{-1}) - 4 experiments; 2-deoxyglucose, sodium fluoride (10^{-2} and 10^{-5} mol.l^{-1}) and 2,4-dinitrophenol (10^{-7} mol.l^{-1}) - 3 experiments.

produced a dose-dependent decrease in ^{14}C-histidine uptake. At all the concentrations used, 10^{-4}, 10^{-5} and 10^{-6} moles.l^{-1}, 2,4-dinitrophenol gave significant inhibition ($p < 0.01$) while antimycin A produced a significant decrease at 10^{-6} moles.l^{-1} ($p < 0.001$) and 10^{-7} moles.l^{-1} ($p < 0.05$).

2.4 Inhibitors of protein, RNA and DNA synthesis

The inhibitor of protein synthesis, cycloheximide, and RNA synthesis, actinomycin D, gave a significant inhibition of ^{14}C-histidine uptake at 10^{-5} and 10^{-6} moles.l^{-1} ($p < 0.01$). Mitomycin C, an inhibitor of DNA synthesis, over the dose range 10^{-5} to 10^{-7} moles.l^{-1} had no significant inhibitory effect (Fig. 18).

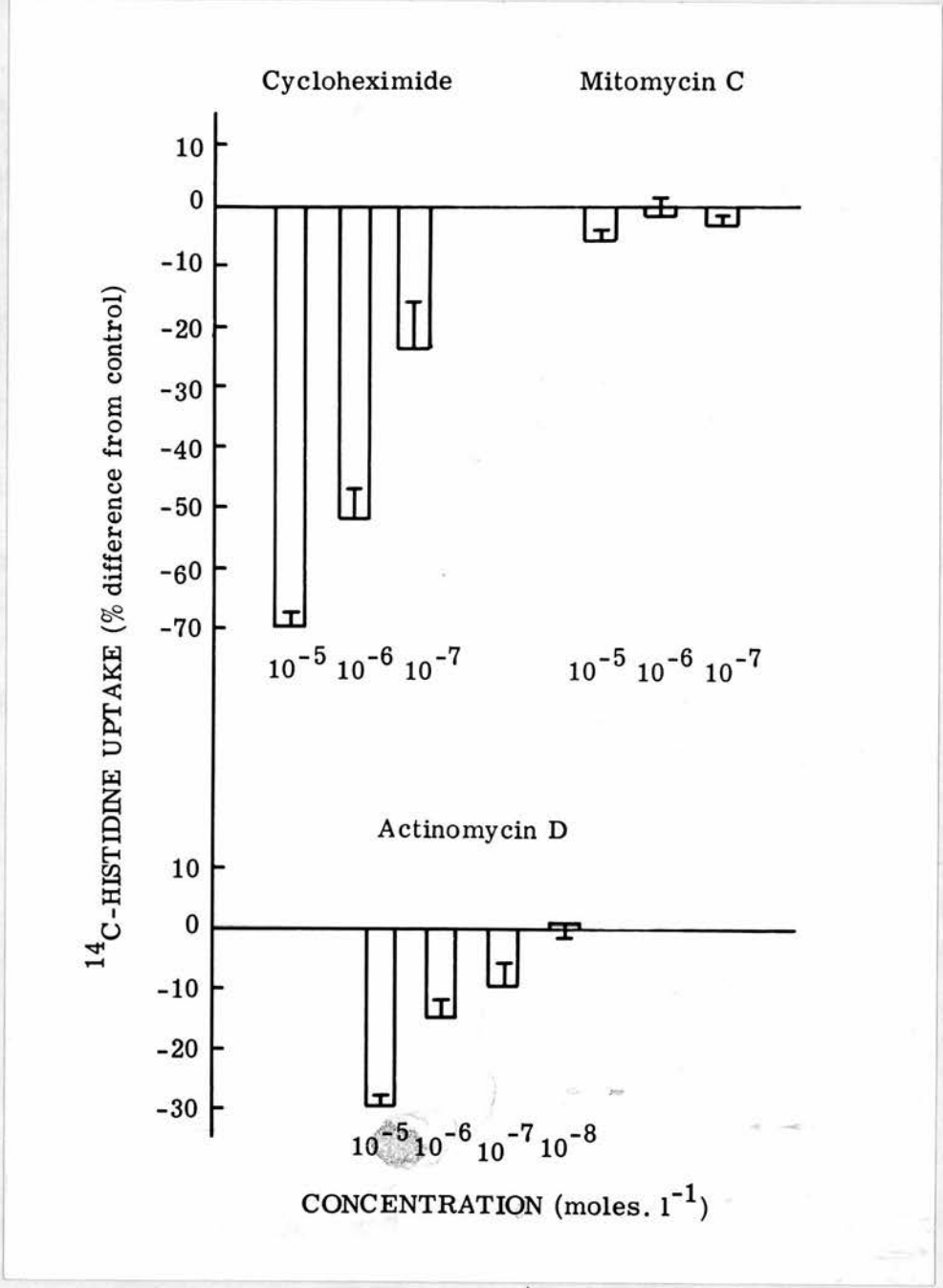

Fig. 18

The effect of metabolic inhibitors on ^{14}C-histidine uptake. Each column represents the mean ± 1 S.E. Actinomycin D (10^{-6} and 10^{-7} mol.l^{-1}) - 5 experiments; cycloheximide and mitomycin C - 4 experiments; actinomycin D (10^{-6} and 10^{-7} mol.l^{-1}) - 5 experiments; cycloheximide and mitomycin C - 4 experiments; actinomycin D (10^{-5} and 10^{-8} mol.l^{-1}) - 3 experiments.

3.0 EFFECT OF METABOLIC INHIBITORS ON HISTAMINE SYNTHESIS

The amount of ^{14}C-histamine formed *de novo* from the ^{14}C-histidine which was incorporated in the presence of the various metabolic inhibitors, expressed as the percentage difference from controls, is shown in Table X.

Only NSD 1055 had any significant effect on the amount of ^{14}C-histamine synthesized. It gave significant inhibition ($p < 0.001$), a greater than 90% reduction in the amount formed, at all concentrations used.

TABLE X

The effect of metabolic inhibitors on the amount of ^{14}C-histamine formed. The figures represent the mean difference from controls (± 1 S.E.) expressed as a percentage. • = 2 experiments; ^{o} = 3 experiments; * = 4 experiments; $^{+}$ = 6 experiments; ** = experiment performed in glucose-free Tyrode's buffer; M = moles.l^{-1}.

AGENT	No. of experiments	10^{-2} M	10^{-3} M	10^{-4} M	10^{-5} M	10^{-6} M	10^{-7} M	10^{-8} M
Iodoacetamide	3			+3.7 ± 9.4	+5.4 ± 18.0	+9.3 ± 14.1	-1.7 ± 1.7	
NSD 1055	4			-98.9 ± 1.1	-97.1 ± 0.2	-91.3 ± 2.2		
Cytochalasin B	5					-8.4 ± 8.8	-5.7 ± 6.9	-2.5 ± 4.3
Colchicine	6			+27.2 ± 12.1	+14.3 ± 5.4	+18.6 ± 6.5		
2-deoxy-glucose**	3		-3.8 ± 13.7	-3.0 ± 2.4	+3.8 ± 18.0			
Sodium fluoride	5	-2.6 ± 8.9	+4.0 ± 7.8	-6.3 ± 3.9	+4.8 ± 5.3			
2,4-dinitrophenol	5			-12.6 ± 9.7*	-13.1 ± 6.5	-2.2 ± 6.8	-13.4 ± 9.8	
Antimycin A	5					+16.7 ± 12.8	+6.2 ± 3.9	+5.1 ± 10.1
Cycloheximide	4				-11.7 ± 7.5	-11.5 ± 3.9	-12.3 ± 6.5	
Mitomycin C	4				+2.1 ± 6.8	-0.2 ± 3.6	+1.1 ± 6.8	
Actinomycin D	5				+16.9 ± 12.6	+6.7 ± 9.4	-4.4 ± 5.4	+1.0 ± 4.0

TABLE X

4.0 EFFECT OF METABOLIC INHIBITORS ON ^{14}C-HISTIDINE UPTAKE

The effect of the various metabolic inhibitors on ^{14}C-histamine uptake is shown in Table XI.

Sodium fluoride at 10^{-2} moles.l^{-1} and antimycin A at 10^{-6} moles.l^{-1} gave slight but significant inhibition ($p < 0.05$) of ^{14}C-histamine uptake. All other agents tested failed to produce a significant effect.

TABLE XI

The effect of metabolic inhibitors on the uptake of ^{14}C-histamine. The figures represent the mean difference from controls (± 1 S.E.) expressed as a percentage. ^{o} = 3 experiments; * = 4 experiments; ** = experiment performed in glucose-free Tyrode's buffer; M = moles.l^{-1}.

AGENT	No. of experiments	10^{-2} M	10^{-3} M	10^{-4} M	10^{-5} M	10^{-6} M	10^{-7} M	10^{-8} M
Iodoacetamide	2			-13.3 ± 6.2	+12.1 ± 5.3	+4.8 ± 0.9		
NSD 1055	3			-17.9 ± 10.2	-7.2 ± 5.7	-5.9 ± 9.8		
Cytochalasin B	5					-0.2 ± 3.0	-7.0 ± 6.9*	-2.3 ± 6.5*
Colchicine	3			-7.1 ± 8.8	-5.4 ± 3.5	-7.1 ± 9.3		
2-deoxy-glucose**	2		+17.1 ± 2.0	-1.5 ± 2.1	+14.3 ± 12.3			
Sodium fluoride	4	-13.2 ± 2.8°	-0.1 ± 4.1	+5.3 ± 7.7				
2,4-dinitrophenol	3			-13.3 ± 5.3	-5.8 ± 5.1	+0.8 ± 7.3		
Antimycin A	3					-19.5 ± 3.6*	-7.1 ± 6.0	-0.7 ± 7.2
Cycloheximide	3				-5.3 ± 8.7	-6.3 ± 4.9	-0.1 ± 3.6	
Mitomycin C	3				-5.1 ± 1.5	-5.6 ± 5.3	-15.1 ± 4.0	
Actinomycin D	2				-3.2 ± 4.6	-2.8 ± 4.6	-0.5 ± 0.9	

TABLE XI

5.0 REQUIREMENT FOR SODIUM IONS

The effect of ouabain, a specific inhibitor of Na^+-K^+-dependent ATPase, on ^{14}C-histidine and ^{14}C-histamine and ^{14}C-histamine formation is shown in Fig. 19.

Ouabain produced a dose-dependent decrease in the amount of ^{14}C-histidine incorporated. There was a significant difference from control at all concentrations used ($p < 0.01$). ^{14}C-histamine formation was also significantly decreased by ouabain at 5×10^{-5} moles.l^{-1} ($p < 0.01$), 10^{-5} and 5×10^{-6} moles.l^{-1} ($p < 0.05$) and 5×10^{-6} moles.l^{-1} ($p < 0.001$).

The percentage difference between the amount of ^{14}C-histamine taken up by the cell suspensions in the presence and absence of ouabain was relatively small although it is statistically significant at 5×10^{-5} and 5×10^{-6} moles.l^{-1} ($p < 0.05$).

Replacing the sodium ions in the buffer by choline resulted in a significant reduction in the amount of ^{14}C-histidine ($p < 0.05$) and ^{14}C-histamine ($p < 0.01$) incorporated (Fig. 19). There was no significant effect on the amount of ^{14}C-histamine formed.

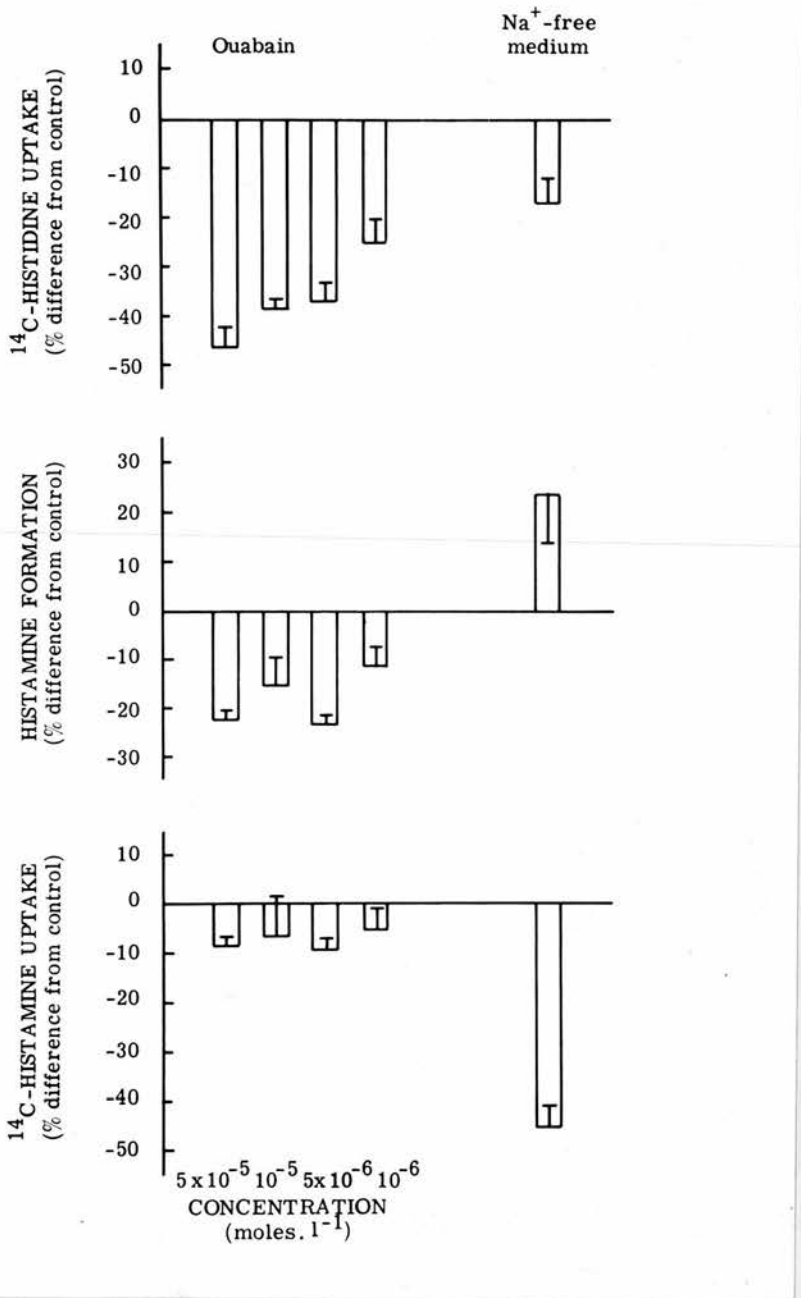

Fig. 19

The effect of ouabain and Na^+-free medium on the uptake of ^{14}C-histidine and ^{14}C-histamine and ^{14}C-histamine formation. Each column represents the mean ± 1S.E. Ouabain on ^{14}C-histidine uptake and ^{14}C-histamine formation (5×10^{-5} and 5×10^{-6} mol.l^{-1}) - 4 experiments; 10^{-5} and 10^{-6} mol.l^{-1} - 5 experiments. Ouabain on ^{14}C-histamine uptake - 3 experiments. Histidine uptake and histamine formation in Na^+-free medium - 5 experiments. Histamine uptake in Na^+-free medium - 4 experiments.

6.0 TIME COURSE OF THE EFFECT OF METABOLIC INHIBITORS

Ouabain and antimycin A were used to study the variation in inhibitory effects with time (Fig. 20).

Ouabain produced no significant decrease in ^{14}C-histidine uptake over the first 30 min of incubation but there was a steady increase in the percentage inhibition as the incubation time was increased. At 120 min the ouabain treated cells incorporated only 49% as much ^{14}C-histidine as the control cells. At this time the cell viabilities were the same in both control and treated suspensions but there was a gradual decrease in the viability of the treated cells after this time. Ouabain did not affect ^{14}C-histamine synthesis up to 120 min. After 120 min there was a decrease in histamine synthesis in treated cells presumably due to loss in viability.

There was a constant degree of inhibition of ^{14}C-histidine uptake by antimycin A throughout the period studied. The percentage decrease in uptake ranged from 34% at 120 min to 38% at 30 min. In this experiment at an incubation time of 30 and 60 min there was a significant increase in histamine formation ($p < 0.05$). Above 60 min there was no significant difference from control.

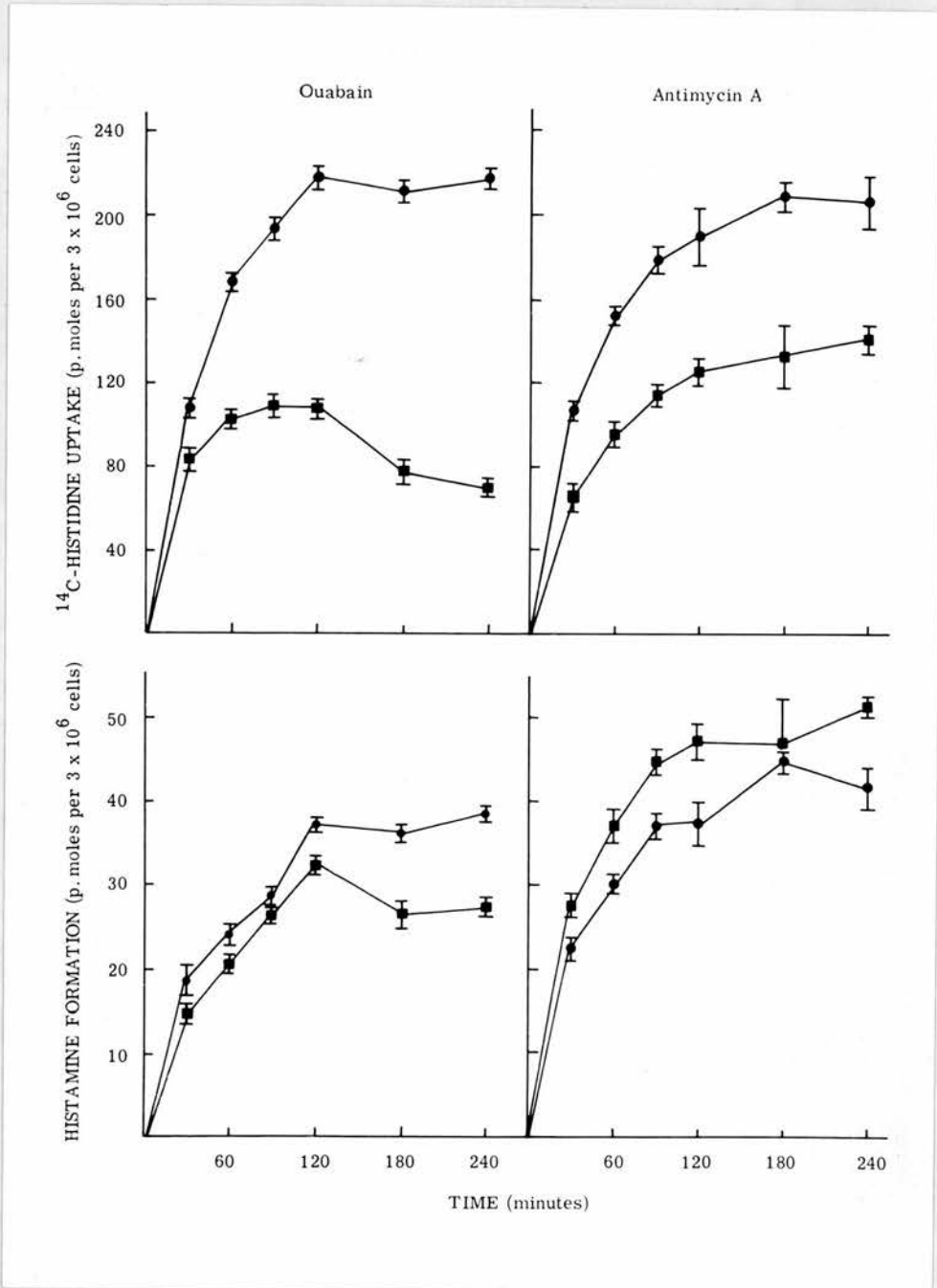

Fig. 20

Time course of the effect of ouabain (10^{-5} mol.l^{-1}) and antimycin A (10^{-6} mol.l^{-1}) on the uptake of ^{14}C-histidine and ^{14}C-histamine formation. The results represent the values from one experiment and the bars represent the mean of the triplicates ± 1 S.E.

7.0 EFFECT OF METABOLIC INHIBITORS ON ^{14}C-HISTIDINE UPTAKE BY MACROPHAGE-RICH, NEUTROPHIL-RICH AND NORMAL BONE MARROW CELL SUSPENSIONS

^{14}C-histidine uptake by macrophage-enriched, neutrophil-enriched and normal bone marrow cell suspensions was inhibited by iodoacetamide, ouabain and cycloheximide (Fig. 21).

Iodoacetamide produced a dose-dependent decrease in ^{14}C-histidine uptake by normal bone marrow cells which was significantly different from the controls at 10^{-4} moles.l^{-1} ($p < 0.001$) and 10^{-5} moles.l^{-1} ($p < 0.01$). The uptake by the same cell type was significantly decreased by ouabain ($p < 0.01$) and cycloheximide ($p < 0.05$) at 10^{-5} moles.l^{-1}.

The highest concentration of iodoacetamide, 10^{-4} moles.l^{-1}, and ouabain at 10^{-5} moles.l^{-1} inhibited ($p < 0.01$) the uptake of ^{14}C-histidine by mononuclear cells. Cycloheximide, at 10^{-5} moles.l^{-1}, also produced a significant ($p < 0.05$) decrease in the amount of ^{14}C-histidine incorporated.

A single experiment using a neutrophil-rich cell suspension produced the same pattern of inhibition as was found with normal bone marrow cells although the degree of inhibition of uptake was less.

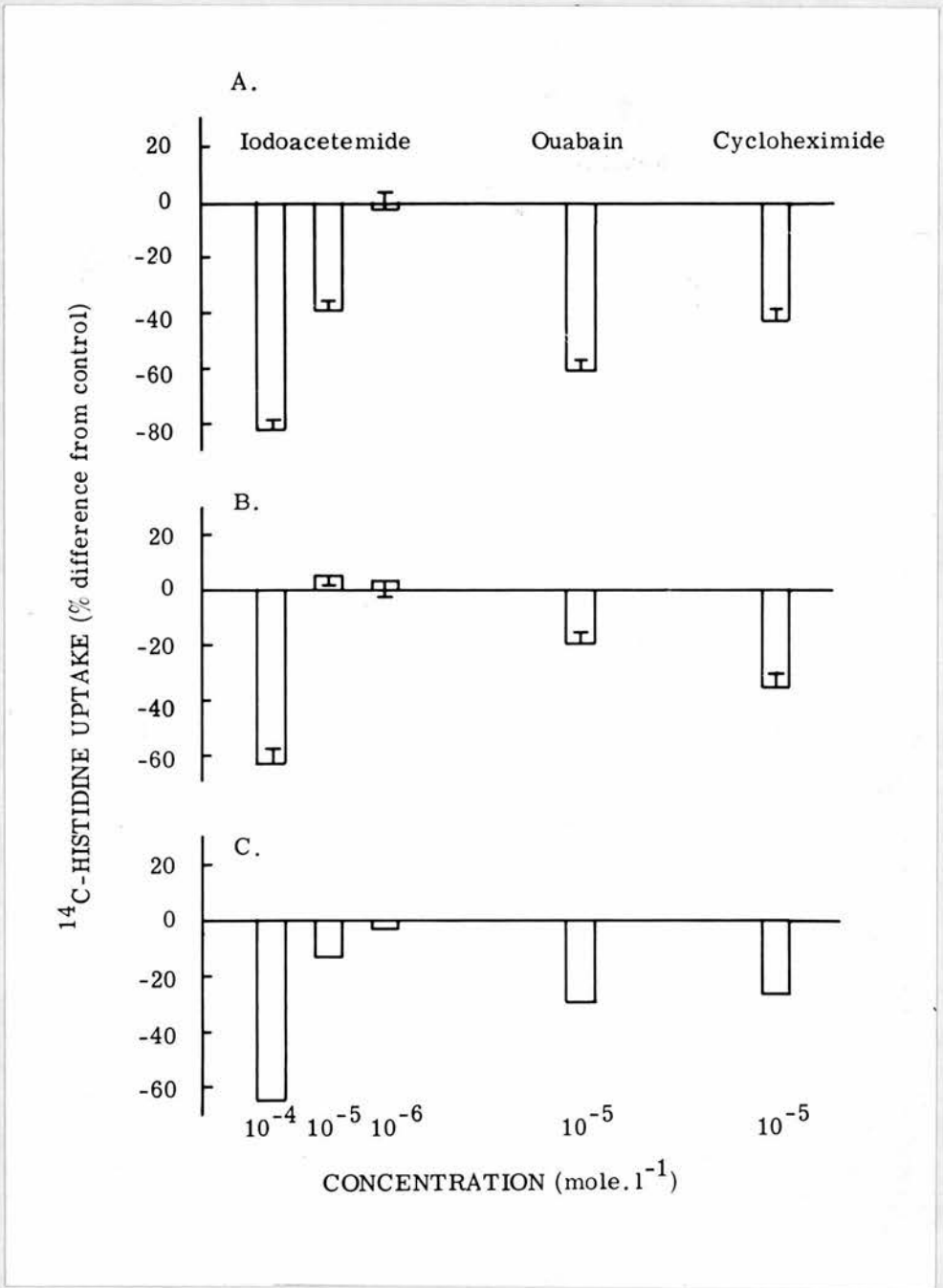

Fig. 21

The effect of metabolic inhibitors on the uptake of ^{14}C-histidine by normal bone marrow, macrophage-enriched and neutrophil-enriched cell suspensions. Each column represents the mean ± 1 S.E. of 3 experiments for normal bone marrow and macrophage-enriched cell suspensions and the results of 1 experiment for the neutrophil-enriched cell suspension.

A = normal bone marrow cells; B = macrophages;

C = neutrophils.

8.0 SUMMARY

^{14}C-histidine uptake by basophil-enriched cell suspensions was decreased by agents which inhibit glycolysis, oxidative phosphorylation, histidine decarboxylase, protein synthesis and RNA synthesis. Inhibition was demonstrable in a dose- and time-dependent fashion and at concentrations which had no apparent effect on cell viability. Inhibitors of DNA synthesis and microtubule function had no effect on ^{14}C-histidine uptake. Cytochalasin B, an inhibitor of microfilament function, also decreased the amount of ^{14}C-histidine incorporated but only at concentrations previously shown to affect hexose transport.

NSD 1055, an histidine decarboxylase inhibitor, gave a significant decrease in the amount of ^{14}C-histamine formed. In the presence of all the other metabolic inhibitors tested, except ouabain, the basophils produced the same total amount of histamine even although less of the substrate, histidine, had been taken up.

^{14}C-histamine uptake was significantly decreased by high concentrations of sodium fluoride and antimycin A.

^{14}C-histidine and ^{14}C-histamine uptake were dependent on the presence of Na^+ ions and an active Na^+-K^+-dependent ATPase.

These results suggest that histidine was taken up by an active-mediated process but that once the histidine had entered the basophil the amount of histamine formed was independent of energy from the main metabolic pathways. Since histamine uptake was relatively small the energy required may be negligible and the uptake continued even in the presence of metabolic inhibitors. There was a requirement for Na^+ ions for maximal histamine incorporation.

SECTION III - STUDIES ON THE BIOREGULATION OF HISTIDINE AND HISTAMINE UPTAKE AND NEW HISTAMINE FORMATION BY BASOPHILS

1.0 INTRODUCTION

Basophil degranulation, whether by humoral or cellular mechanisms, leads to the release of several pharmacologically-active mediators including histamine (Lichtenstein, 1968). Histamine has been shown to interact with the cell type from which it originates. For instance, the antigen-induced release of histamine from basophils of sensitized individuals was inhibited by the addition of histamine prior to antigen challenge (Bourne et al, 1971). Low concentrations of histamine inhibit the chemotactic response of normal basophils to a fragment cleaved from the fifth component of complement, C5a (Lett-Brown and Leonard, 1977).

In addition to the "classical" pharmacological actions of histamine, such as increased vascular permeability, contraction of smooth muscle, bronchoconstriction and the secretion of gastric hydrochloric acid (Beaven, 1976), it is now recognized that it has various other effects such as those on leucocytes. For example, histamine promotes the migration of human eosinophils in vivo (Clark et al, 1975) and of guinea pig eosinophils in vitro (Jones and Kay, 1977). Histamine also inhibits eosinophil "chemotaxis" to endotoxin-activated serum at 10^{-5} moles.l^{-1} but enhances it at 10^{-6} moles.l^{-1} (Clark et al, 1977). Furthermore, human eosinophil complement receptors were enhanced, or unfolded, by histamine (Anwar and Kay, 1978) and the amine inhibits lysosomal enzyme release from neutrophils (Busse and Sosman, 1976).

The pharmacological actions of histamine are mediated by at least two distinct classes of receptor designated H1 and H2, which can be differentiated by the use of receptor specific antagonists. The effects on both the vasculature and smooth

muscle are blocked by classical, H1, histamine receptor antagonists (Ash and Schild, 1966), whereas gastric acid secretion is abrogated by the more recently developed H2-receptor antagonists (Black et al, 1972). The differentiations of these two receptor types has also been confirmed by the relative agonist activities of certain histamine analogues (Durant et al, 1975; Parsons et al, 1977).

Since histamine has been shown to influence many cellular processes its effect on its own biosynthesis was investigated by studying its effect on ^{14}C-histidine and ^{14}C-histamine uptake and ^{14}C-histamine de novo. In addition the effects of the major in vivo histamine catabolites and various histamine receptor antagonists and agonists were studied.

2.0 THE EFFECT OF HISTAMINE

The effect of histamine and its major catabolites, imidazoleacetic acid (ImAA), 1-methyl-4-imidazoleacetic acid (1,4-MeImAA), N-acetylhistamine (N-AcHm) and 1,4-methylhistamine (1,4-MeHm), on ^{14}C-histidine and ^{14}C-histamine uptake and ^{14}C-histamine formation is shown in Figs. 22-24. Histamine receptor agonists and antagonists were then used to identify the possible mechanism of histamine's effect.

2.1 Effect of histamine and its major metabolites on ^{14}C-histidine and ^{14}C-histamine uptake and ^{14}C-histamine formation

Histamine and two of its catabolites, ImAA and 1,4-MeImAA, increased the uptake of ^{14}C-histidine (Fig. 22). Histamine significantly increased the amount of ^{14}C-histidine taken up at 10^{-3} moles.l^{-1} ($p < 0.001$) and 10^{-4} moles.l^{-1} ($p < 0.01$). 1,4-MeImAA gave a significant effect at 10^{-3} moles.l^{-1} ($p < 0.01$) while at 10^{-3} and 10^{-5} moles.l^{-1} ImAA produced a significant enhancement ($p < 0.01$). N-AcHm and 1,4-MeHm had no appreciable effect over the concentration range 10^{-3} to 10^{-5} moles.l^{-1}.

Histamine, at 10^{-3} and 10^{-4} moles.l^{-1}, produced a significant enhancement of histamine formation ($p < 0.001$) while ImAA at 10^{-3} moles.l^{-1} also produced an increase ($p < 0.05$). 1,4-MeImAA, N-AcHm and 1,4-MeHm had no apparent effect on histamine formation under the conditions used.

In other systems histamine had been shown to produce pposite effects at different concentrations. However, between 10^{-5} and 10^{-10} moles.l^{-1} histamine failed to influence significantly ^{14}C-histidine uptake or ^{14}C-histamine formation.

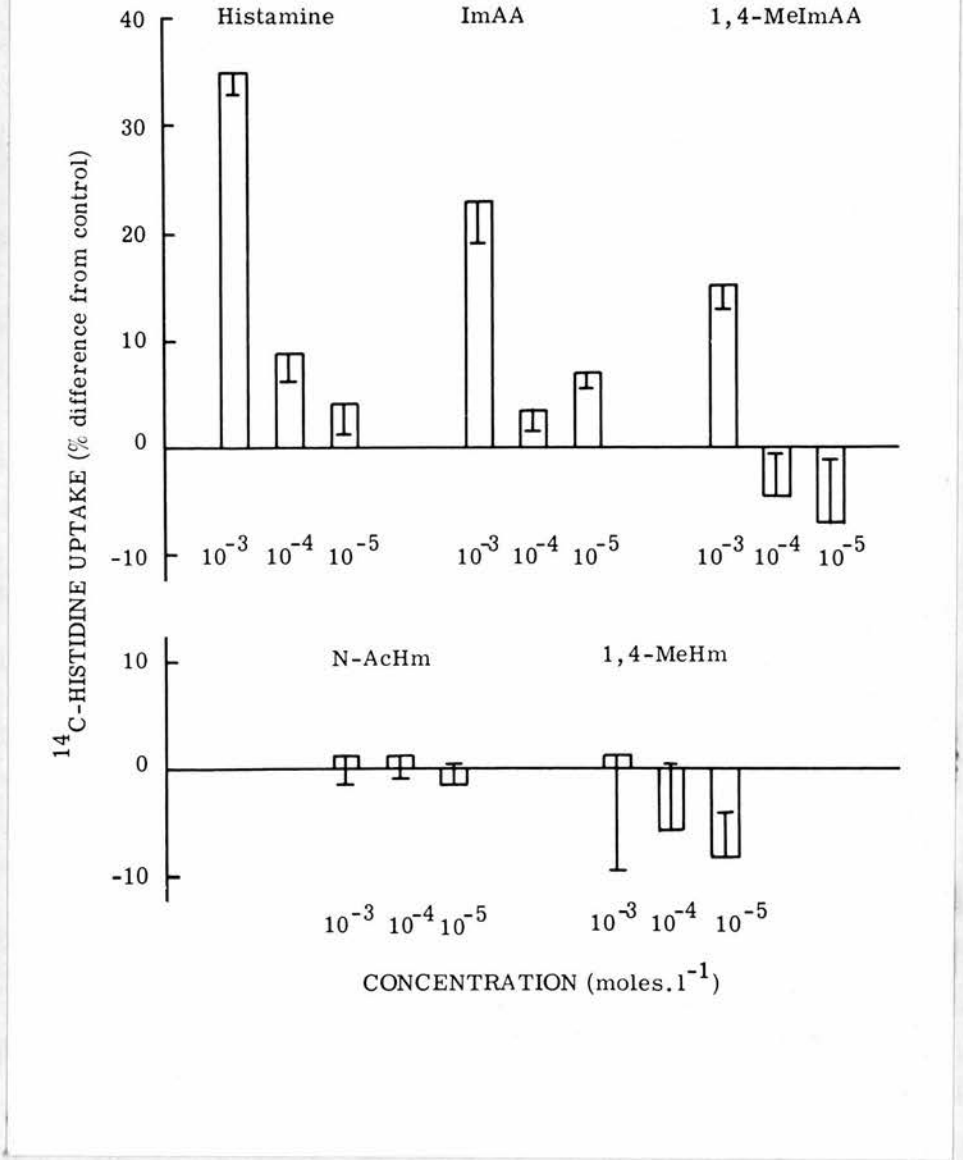

Fig. 22

The effect of histamine and its major catabolites on ^{14}C-histidine uptake. Each column represents the mean ± 1 S.E. Histamine - 13 experiments; ImAA and N-AcHm - 5 experiments; 1,4-MeHm and 1,4-MeImAA - 6 experiments.

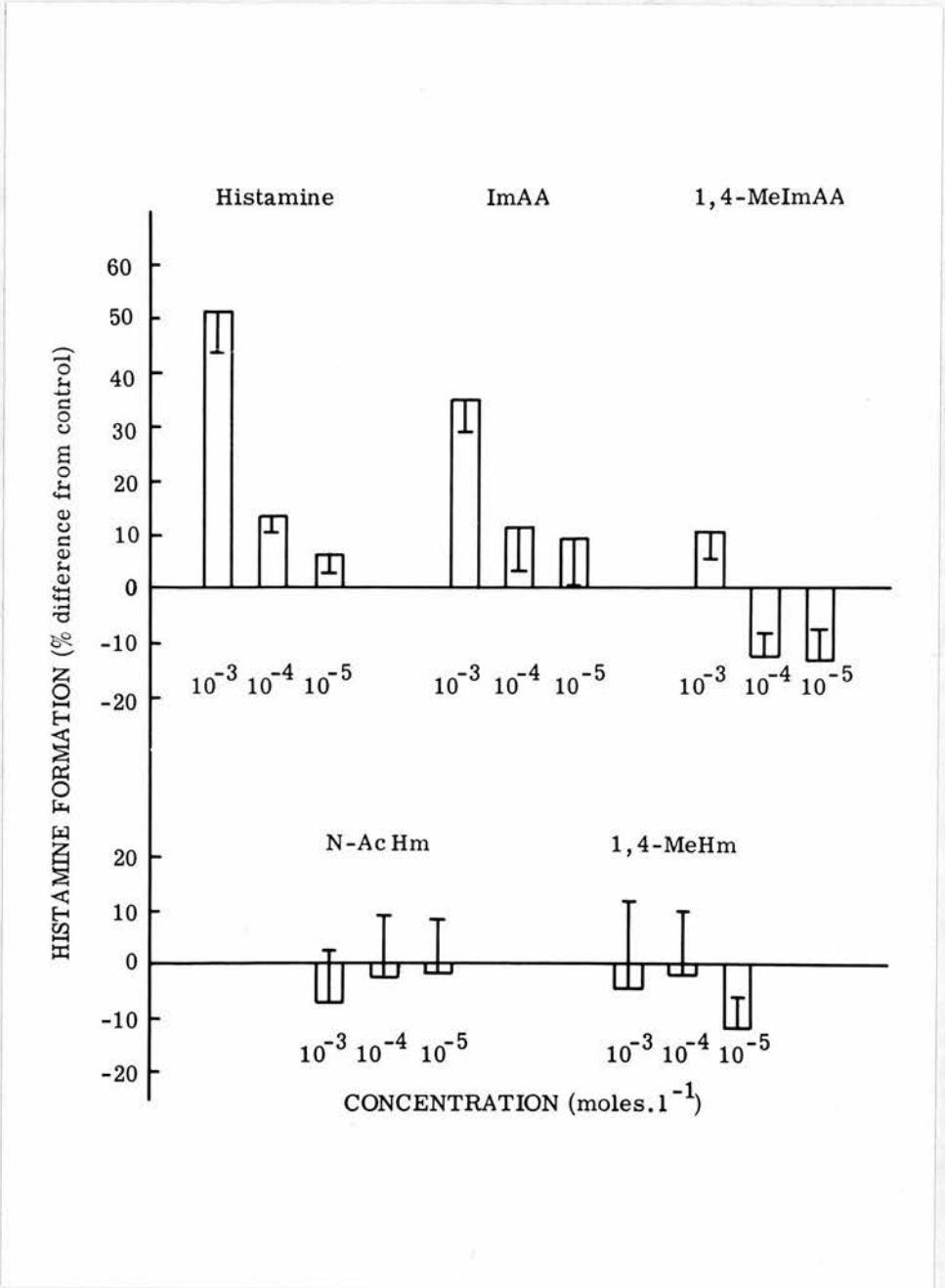

Fig. 23

The effect of histamine and its major catabolites on ^{14}C-histamine formation. Each column represents the mean ± 1 S.E. Histamine - 13 experiments; 1,4-MeHm and 1,4-MeImAA - 6 experiments; N-AcHm - 5 experiments; ImAA - 4 experiments.

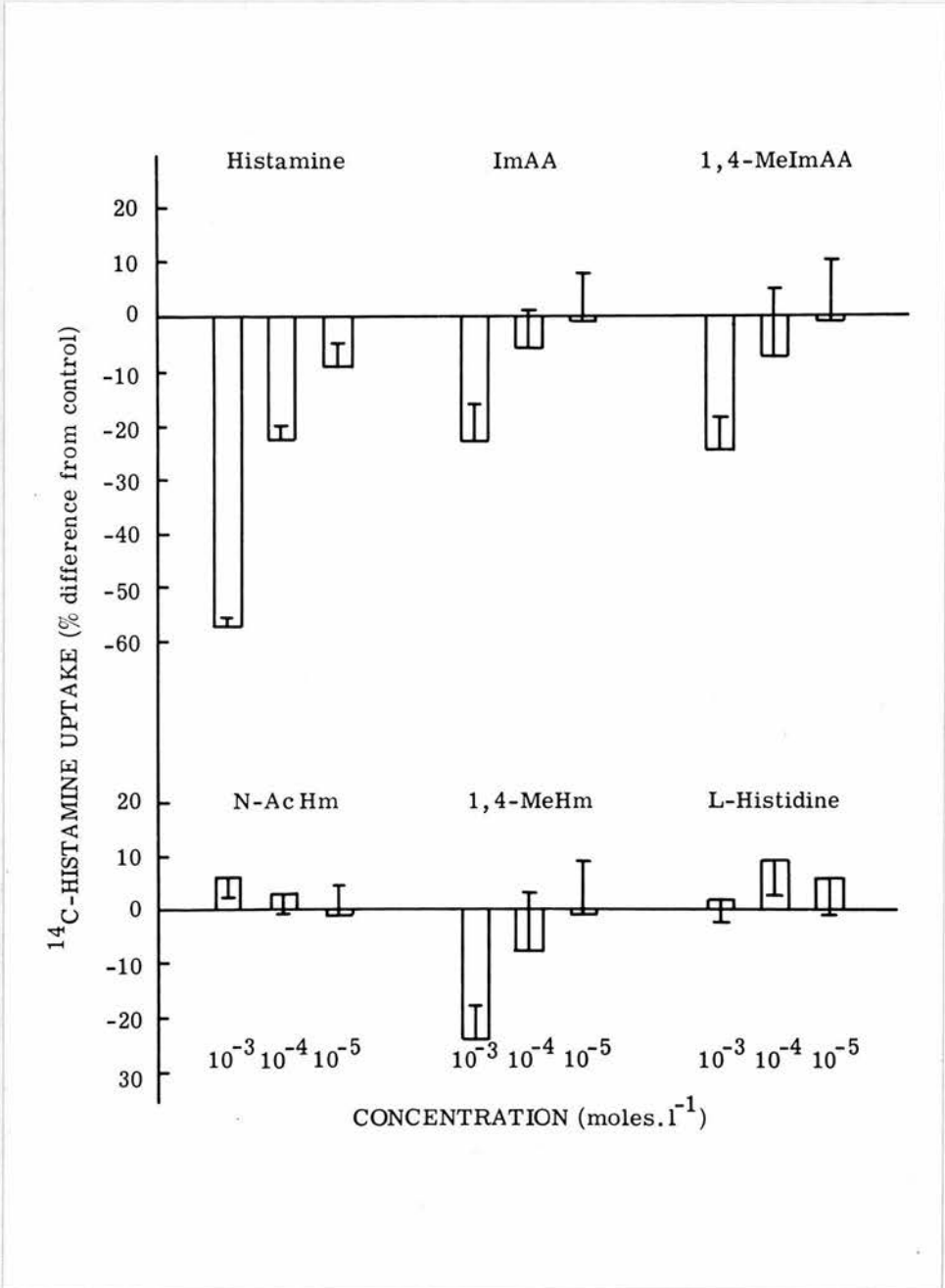

Fig. 24

The effect of histamine and its major metabolites on the uptake of ^{14}C-histamine. Each column represents the mean ± 1 S.E. L-histidine - 6 experiments; Hm (10^{-4} and 10^{-5} mol.l^{-1}), ImAA, 1,4-MeImAA and 1,4-MeHm - 4 experiments; N-AcHm - 3 experiments; Hm (10^{-3} mol.l^{-1}) - 2 experiments.

As expected histamine significantly inhibited the uptake of ^{14}C-histamine at 10^{-3} and 10^{-4} moles.l^{-1} ($p < 0.001$). The decrease in the uptake of ^{14}C-histamine was dose-dependent as was the inhibition caused by ImAA, 1,4-MeImAA and 1,4-MeHm which was statistically significantly different from control values at 10^{-3} moles.l^{-1} ($p < 0.05$). L-histidine and N-AcHm had no effect on ^{14}C-histamine uptake.

2.2 Time course

The effect of varying the time of incubation with ^{14}C-histidine, after pre-incubation for various times with histamine, on ^{14}C-histidine uptake is shown in Fig. 25. With all pre-incubation regimes there was an increase in ^{14}C-histidine uptake, compared to controls, which become more marked with time. There was a statistically significant increase ($p < 0.05$) at 30, 60, 90 and 120 min with a 40 min pre-incubation with histamine. However, the increase did not become significant until 60 min when a shorter, 20 min, pre-incubation was used and until 90 min when the pre-incubation with histamine was only 10 min or the ^{14}C-histidine and histamine were added together.

The time course for the increase of histamine formation followed the same pattern as that for ^{14}C-histidine uptake. Pre-incubation with histamine for 40 min produced an increase ($p < 0.05$) in the amount of histamine formed at times above 60 min. However, when the histamine and ^{14}C-histidine were added simultaneously there was no significant effect until 90 min.

The length of the pre-incubation with histamine prior to the addition of ^{14}C-histidine for 90 min did not appreciably

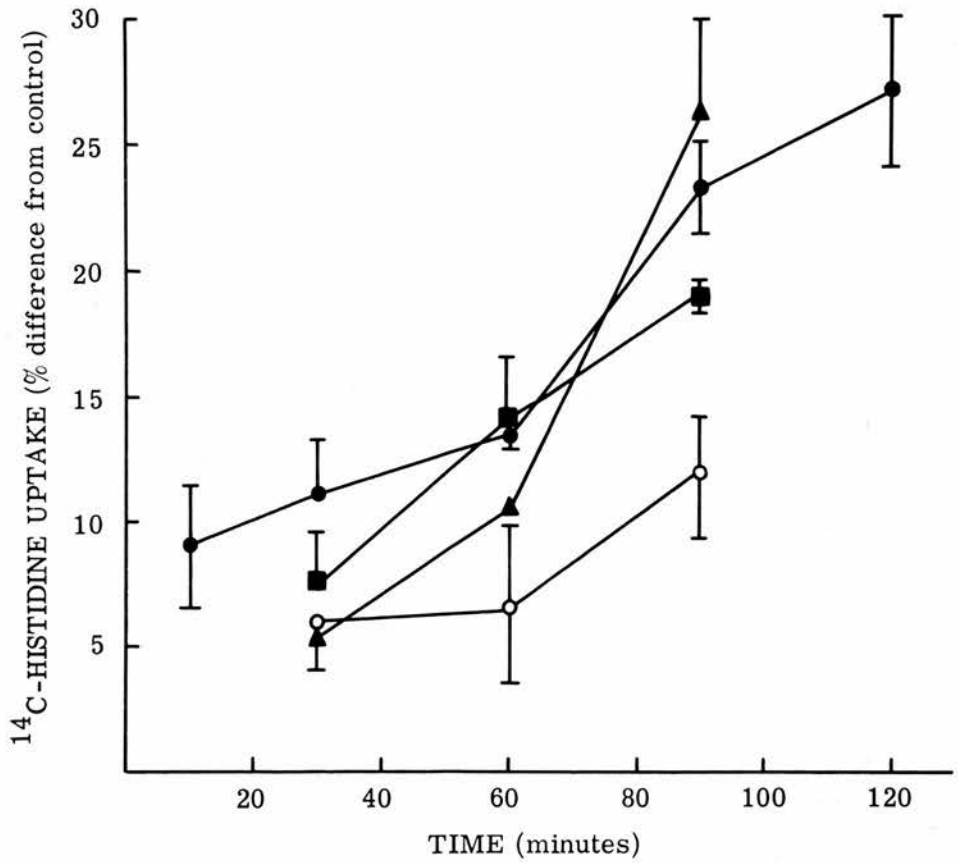

Fig. 25

Time course of ^{14}C-histidine uptake following pre-incubation for 40 min (● —●), 20 min (■ — ■), 10 min (▲ — ▲) or no pre-incubation (O — O) with histamine (10^{-3} moles.1^{-1}). The results represent the mean ± 1 S.E. 40 min pre-incubation at 30, 60 and 90 min - 4 experiments; at 10 and 120 min - 3 experiments; 20 and 10 min pre-incubation - 3 experiments; no pre-incubation - 4 experiments.

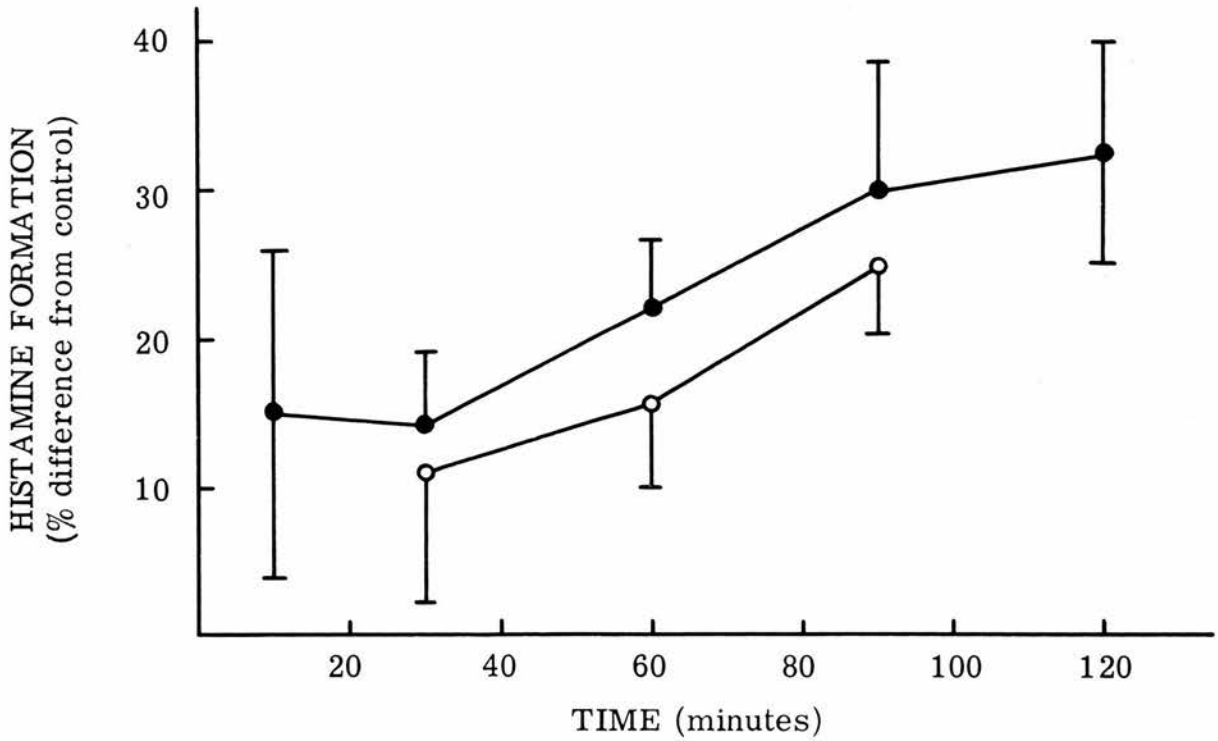

Fig. 26

Time course of ^{14}C-histamine formation following pre-incubation for 40 min (● — ●) or no pre-incubation (O — O) with histamine (10^{-3} moles.l^{-1}). The results represent the mean ± 1 S.E. 40 min pre-incubation at 30 and 90 min - 4 experiments; at 10, 60 and 120 min - 3 experiments; no pre-incubation - 3 experiments.

alter the enhancement of ^{14}C-histidine uptake (Fig. 26 and Table XII) nor histamine formation (Table XII). The histamine must be present during the incubation with ^{14}C-histidine since there was no significant difference from controls in experiments where the histamine was removed by washing prior to the addition of ^{14}C-histidine (Table XII).

TIME OF INCUBATION WITH HISTAMINE (minutes)	UNWASHED		WASHED	
	^{14}C-HISTIDINE UPTAKE (% difference from control)	HISTAMINE FORMATION (% difference from control)	^{14}C-HISTIDINE UPTAKE (% difference from control)	HISTAMINE FORMATION (% difference from control)
60	+27.0 ●	+40.3 ●	-4.5 ●	-2.5 ø
40	+27.2 ●	+42.6 ●	+4.4 ○	-4.8 ●
30	+21.3 ø	+45.6 ø	+0.5 ●	+10.0 ø
20	+18.9 ●	+35.6 ●	+3.9 ●	+10.0 ø
10	+26.3 ●	+45.8 ●	-1.5 ○	-0.2 ø
0	+11.8 ○	+24.8 ●	+7.9 ø	

TABLE XII

The effect of varying the time of incubation with histamine (10^{-3} moles.l^{-1}) and removing the histamine, by washing prior to the addition of ^{14}C-histidine, on the uptake of ^{14}C-histidine and ^{14}C-histamine formation. The figures represent the mean of: 2 ●, 3 ○ experiments or the result of 1 ø experiment.

3.0 THE EFFECT OF HISTAMINE RECEPTOR AGONISTS

2-(2-aminoethyl) thiazole, an H1-receptor agonist, significantly inhibited ^{14}C-histidine uptake at 10^{-3} moles.l^{-1} ($p < 0.01$) and 10^{-4} moles.l^{-1} ($p < 0.05$), and ^{14}C-histamine formation at 10^{-3} moles.l^{-1} ($p < 0.01$) and 10^{-4} and 10^{-5} moles.l^{-1} ($p < 0.05$) (Fig. 27).

"Dimaprit", an H2-receptor agonist, significantly decreased the amount of ^{14}C-histidine incorporated at the highest concentration used, 10^{-4} moles.l^{-1} ($p < 0.01$) and the amount of ^{14}C-histamine formed at all concentrations used ($p < 0.05$). The uptake of ^{14}C-histidine was not significantly affected by 4-methylhistamine, another H2-receptor agonist, but the two lowest doses gave a significant ($p < 0.05$) reduction in the amount of ^{14}C-histamine formed.

2-(2-aminoethyl) thiazole produced a dose-dependent decrease in the amount of ^{14}C-histamine incorporated. The decrease was significantly different from controls at 10^{-3} and 10^{-4} moles.l^{-1} ($p < 0.05$). The H2-receptor agonists were without effect (Fig. 28).

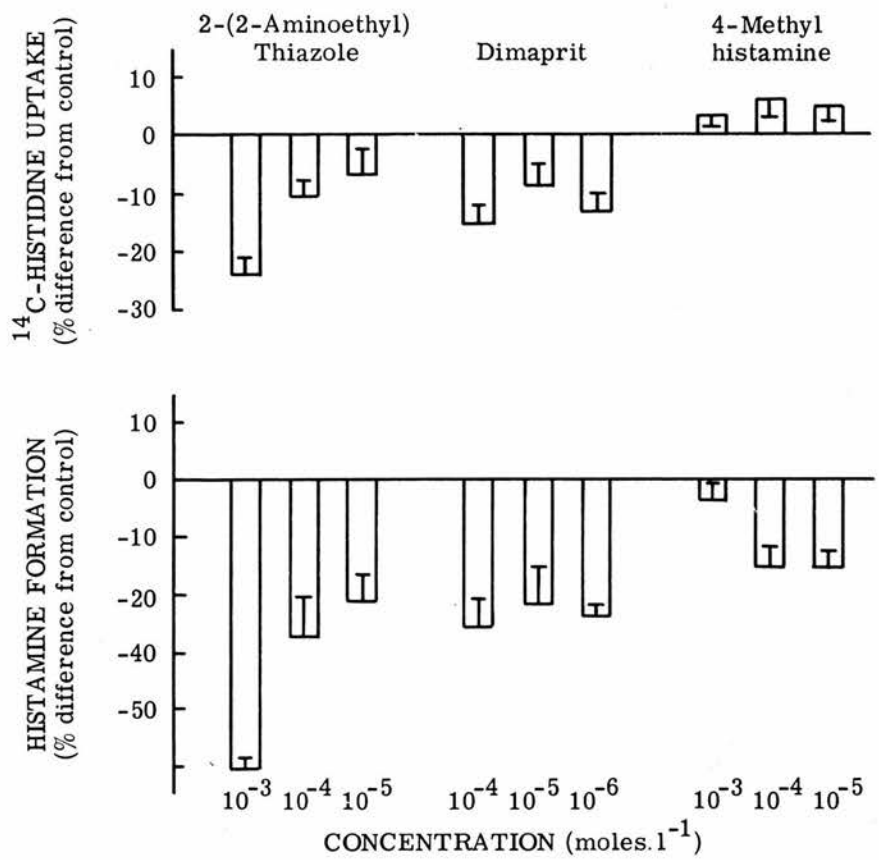

Fig. 27

The effect of histamine agonists on the uptake of ^{14}C-histidine and ^{14}C-histamine formation. Each column represents the mean ± 1 S.E. 2-(2-aminoethyl) thiazole and 4-methyl-histamine and "Dimaprit" (10^{-4} and 10^{-5} moles.l^{-1}) - 4 experiments; "Dimaprit" (10^{-6} moles.l^{-1}) - 2 experiments.

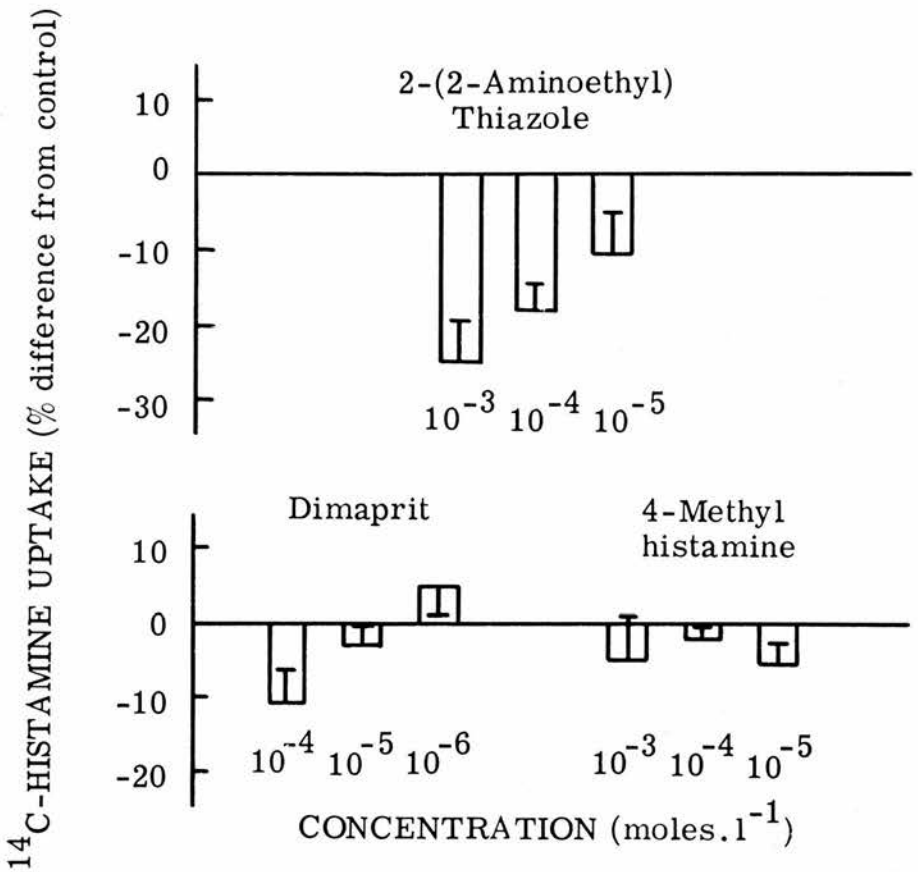

Fig. 28

The effect of histamine agonists on the uptake of ^{14}C-histamine. Each column represents the mean ± 1 S.E. of 4 experiments.

4.0 EFFECT OF HISTAMINE RECEPTOR ANTAGONISTS

Neither the H1-receptor antagonists, mepyramine and chlorpheniramine, nor the H2-receptor antagonists, metiamide and burimamide, had a significant effect on the uptake of ^{14}C-histidine (Fig. 29) and ^{14}C-histamine (Fig. 31) and the formation of ^{14}C-histamine *de novo* (Fig. 30).

Mepyramine and chlorpheniramine at 10^{-5} and 10^{-4} moles.l^{-1}, respectively, gave a significant inhibition ($p < 0.05$) of the enhancement of ^{14}C-histidine uptake due to histamine (Fig. 29). The increase in ^{14}C-histamine formation caused by histamine was significantly inhibited by mepyramine, at 10^{-5} moles.l^{-1} ($p < 0.01$) and at 10^{-6} and 10^{-7} moles.l^{-1} ($p < 0.05$), and chlorpheniramine, at 10^{-4} moles.l^{-1} ($p < 0.05$). In contrast, the H2-receptor antagonists had no significant effect on the histamine-induced enhancement of ^{14}C-histidine uptake or ^{14}C-histamine formation.

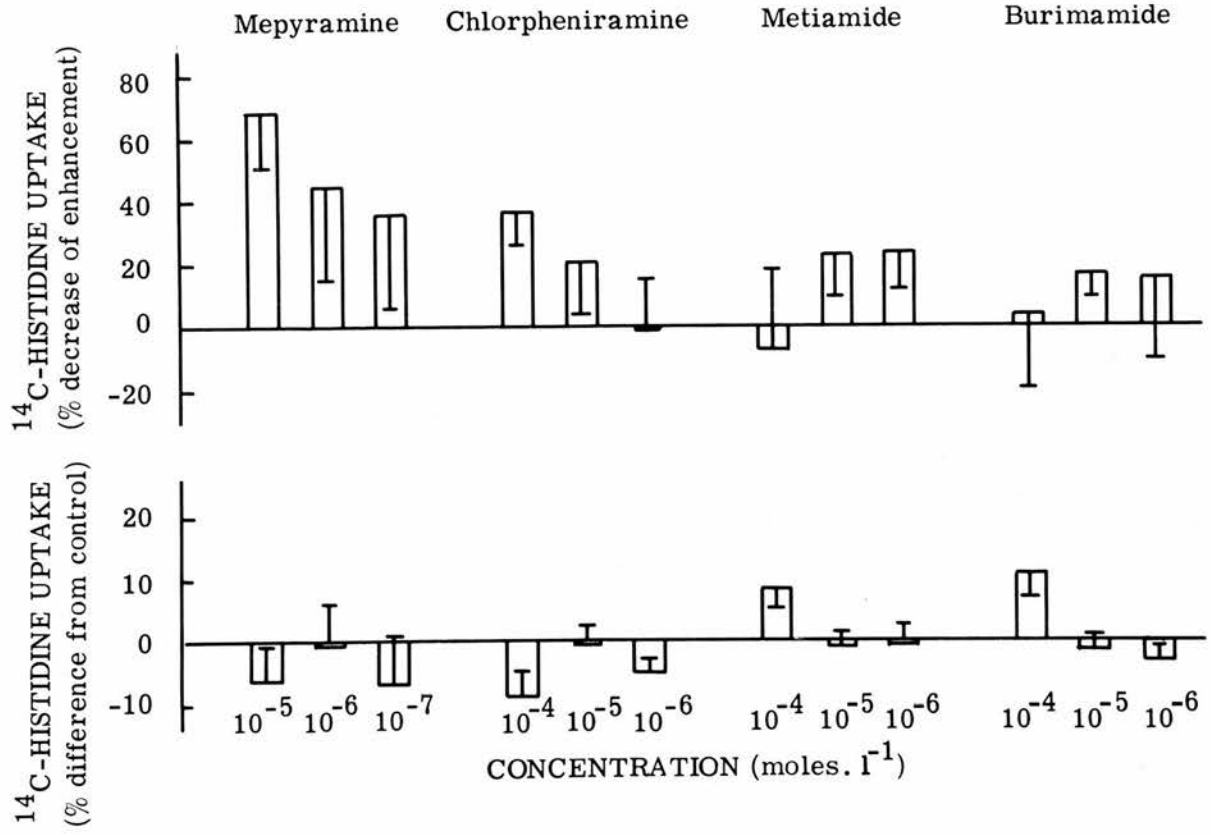

Fig. 29

The effect of histamine antagonists on the uptake of ^{14}C-histidine and the enhancement of ^{14}C-histidine uptake by histamine. The results represent the mean ± 1 S.E.

Enhancement of ^{14}C-histidine: mepyramine and burimamide - 4 experiments; chlorpheniramine and metiamide - 5 experiments.

Uptake of ^{14}C-histidine uptake: chlorpheniramine and metiamide - 7 experiments; burimamide and mepyramine (10^{-5} and 10^{-6} moles.l^{-1}) - 6 experiments; mepyramine (10^{-7} moles.l^{-1}) - 4 experiments.

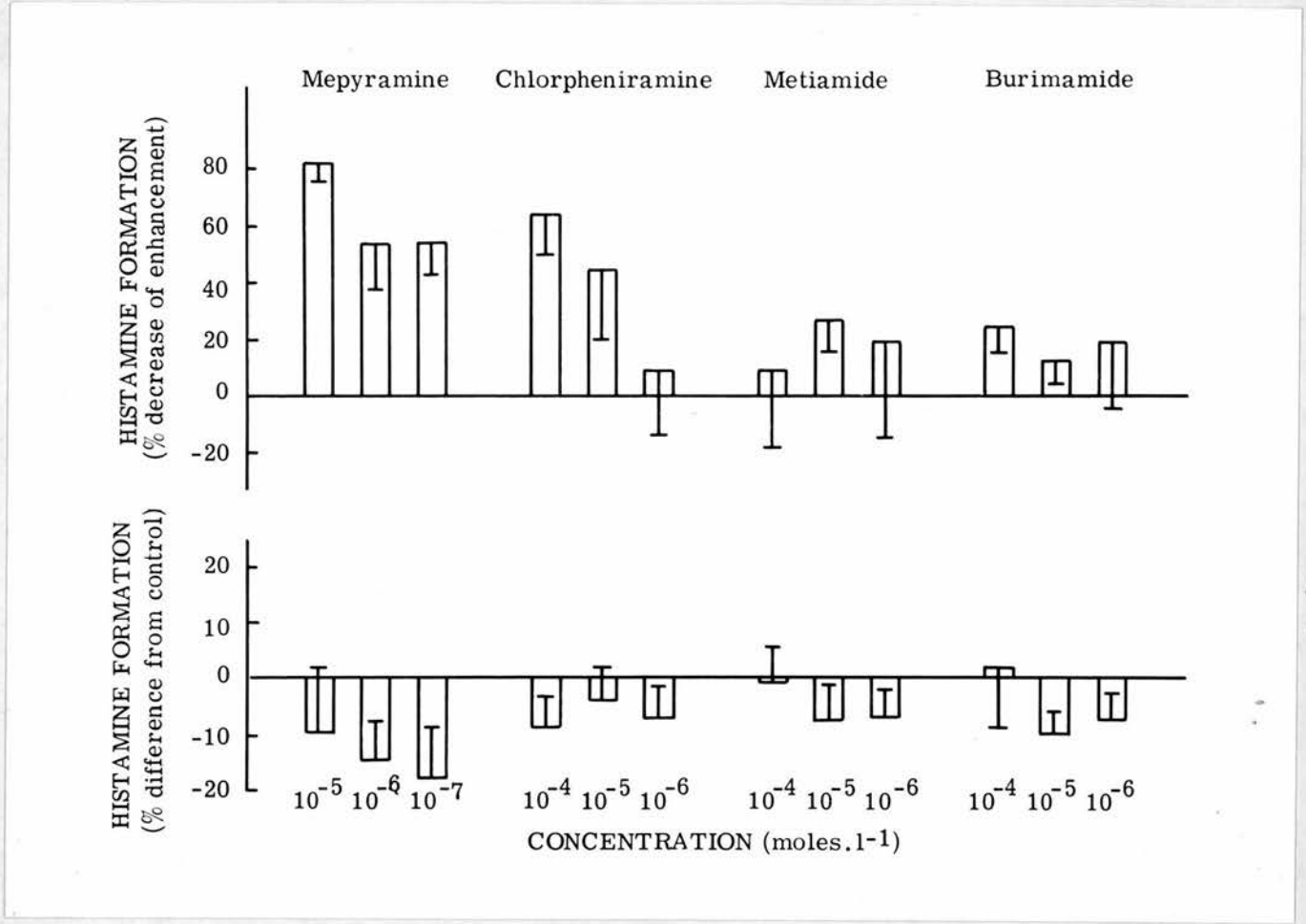

Fig. 30

The effect of histamine antagonists on ^{14}C-histamine formation and the enhancement of ^{14}C-histamine formation by histamine. The results represent the mean ± 1 S.E.

Enhancement of ^{14}C-histamine formation - 4 experiments.

Uptake of ^{14}C-histamine: chlorpheniramine and metiamide - 6 experiments; mepyramine (10^{-5} and 10^{-6} moles.l^{-1}) - 5 experiments; burimamide and mepyramine (10^{-7} moles.l^{-1}) - 4 experiments.

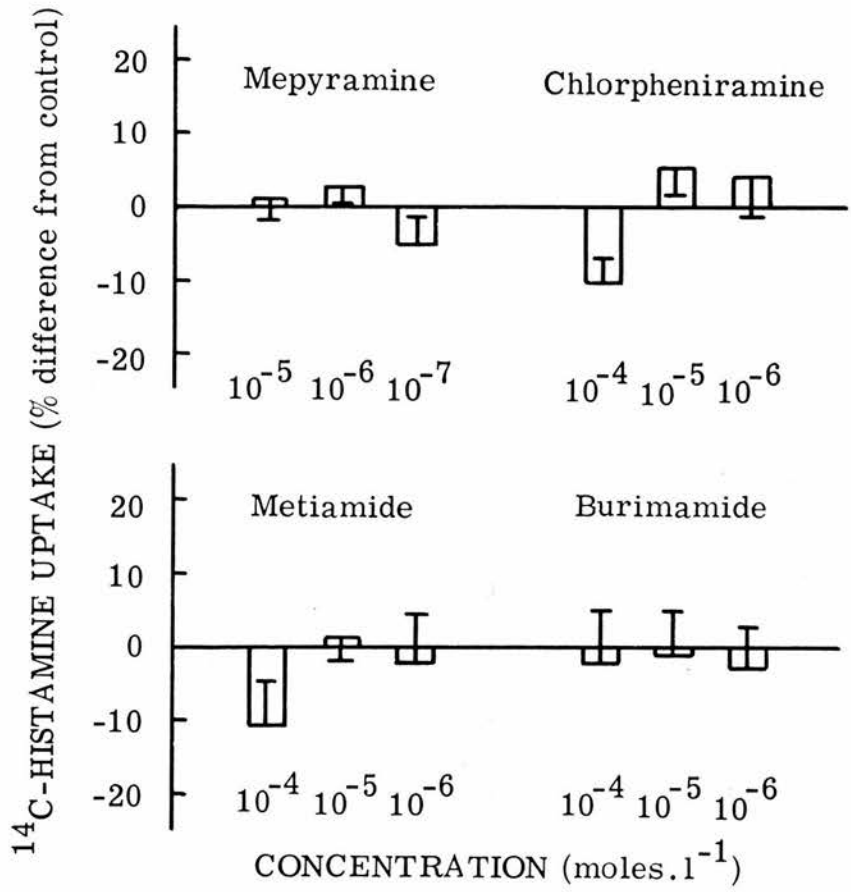

Fig. 31

The effect of histamine antagonists on ^{14}C-histamine uptake. Each column represents the mean ± 1 S.E. of 3 experiments.

5.0 SUMMARY

Histamine increased, in a dose- and time-dependent fashion, the uptake of ^{14}C-histidine and the amount of ^{14}C-histamine formed from the ^{14}C-histidine which had been incorporated. At high concentrations ImAA and 1,4-MeImAA also produced an increased uptake of ^{14}C-histidine and ^{14}C-histamine formation. N-AcHm and 1,4-MeHm had no effect on either of these variables.

^{14}C-histamine uptake was inhibited by histamine, ImAA, 1,4-MeImAA and 1,4-MeHm in a dose-dependent fashion but was unaffected by L-histidine and N-AcHm.

The effect of histamine on ^{14}C-histidine uptake and ^{14}C-histamine formation was abolished by washing the cells free of the added amine prior to the addition of radiolabelled histidine.

^{14}C-histidine uptake and ^{14}C-histamine formation were decreased by the H1-receptor agonist, 2-(2-aminoethyl) thiazole; "Dimaprit" and 4-methylhistamine, H2-receptor agonists, inhibited ^{14}C-histamine formation while only the former affected ^{14}C-histidine uptake.

H1- and H2-receptor antagonists had no significant effect on the variables measured *per se*. However, the H1-receptor antagonists inhibited the histamine-induced increase of ^{14}C-histidine uptake and ^{14}C-histamine formation suggesting that the observed enhancement may be H1-receptor-dependent.

SECTION IV - THE EFFECT OF VARIOUS PHARMACOLOGICAL MEDIATORS AND ANTI-ALLERGY AND ANTI-INFLAMMATORY DRUGS ON ^{14}C-HISTIDINE AND ^{14}C-HISTAMINE UPTAKE AND ^{14}C-HISTAMINE FORMATION

1.0 INTRODUCTION

The challenge of sensitized mast cells and basophils by specific antigen is accompanied by the release of a number of mediators with varied pharmacological effects. Besides histamine and slow reacting substance of anaphylaxis (SRS-A) these agents also include eosinophil chemotactic factor of anaphylaxis (ECF-A) (Kay and Austen, 1971) whose activity has been attributed, at least in part, to two related acidic tetrapeptides, alanine-glycine-serine-glutamic acid and valine-glycine-serine-glutamic acid (Goetzl and Austen, 1975). Lung tissue and blood leucocytes from various species are also capable of synthesizing and releasing prostaglandins (Dawson et al, 1976; Piper and Walker, 1973), bradykinin (Austen and Orange, 1975) and 5-hydroxytryptamine (Sjoerdsma et al, 1975).

Prostaglandins (Bourne et al, 1974) and 5-hydroxytryptamine (Holroyde and Eyre, 1976) have been shown to modulate the release of histamine through modulations of cyclic nucleotide levels. Bradykinin increases cyclic nucleotide levels in guinea pig lung (Stoner et al, 1973) although a direct action on histamine release has not yet been shown.

Anti-inflammatory and anti-allergy drugs are known to influence mast cells and the release of mediators in addition to a number of other cellular processes.

Non-steroidal anti-inflammatory drugs, including aspirin, indomethacin and paracetamol, inhibit prostaglandin synthetase, a collection of microsomal enzymes which convert arachidonic acid to certain prostaglandins by an oxygenation process (Vane, 1971). They are also known to suppress antigen and compound 48/80-induced histamine release from rat mast cells (Norn, 1971).

Adrenocortical steroids with glucocorticoid effects reduce the number of identifiable tissue mast cells (Asboe-Hansen, 1952). They have also been shown to reduce the number of circulating eosinophils, a response which was once used as a test for adrenocortical insufficiency (Thorn et al, 1948). Corticosteroids have little or no effect on prostaglandin synthetase but are thought to inhibit prostaglandin release (Lewis and Piper, 1975). Since they also stabilize lysosomal membranes they may prevent the production of the substrates for prostaglandin synthesis.

The anti-allergy drug, disodium cromoglycate ("Intal"), inhibits the liberation, but not the effect, of mediators of immediate-type hypersensitivity reactions initiated by antibody-antigen interactions in man (Cox, 1967).

Since these drugs affect the function of mast cells and/or basophils and a number of mediators have been shown to influence the release process, their effect on ^{14}C-histidine and ^{14}C-histamine uptake and ^{14}C-histamine formation was investigated.

2.0 EFFECT OF MEDIATORS

The effects of the human ECF-A tetrapeptides and a synthetic analogue, prostaglandins E_1, E_2 and $F_{2\alpha}$, 5-hydroxytryptamine and bradykinin on ^{14}C-histidine and ^{14}C-histamine uptake and ^{14}C-histamine synthesis are shown in Fig. 32-34.

2.1 ECF-A tetrapeptides

The alanyl- (Ala-Gly-Ser-Glu), valyl- (Val-Gly-Ser-Glu) and the analogue (valine-glycine-aspartic acid-glutamic acid) tetrapeptides had no statistically significant effect on the measured parameters (Fig. 32).

2.2 Prostaglandins

The amount of ^{14}C-histidine incorporated and ^{14}C-histamine formed de novo was increased, in a dose-dependent fashion, by the three prostaglandins tested (Fig. 33). There was a significant enhancement ($p < 0.05$) of ^{14}C-histidine uptake by prostaglandins E_2 and $F_{2\alpha}$ at 10^{-4} moles.l^{-1}. Prostaglandin E_1 gave a significant increase in the amount of ^{14}C-histamine synthesized at 10^{-4} and 10^{-5} moles.l^{-1} ($p < 0.05$) and at 10^{-6} moles.l^{-1} ($p < 0.01$). At all the concentrations used, 10^{-4} and 10^{-6} moles.l^{-1} ($p < 0.05$) and 10^{-5} moles.l^{-1} ($p < 0.01$), prostaglandin E_2 increased the amount of ^{14}C-histamine formed. With prostaglandin $F_{2\alpha}$ the amount of ^{14}C-histamine formed was significantly different from control values at 10^{-4} and 10^{-5} moles.l^{-1} ($p < 0.05$).

There was no marked effect on ^{14}C-histamine uptake by any of the prostaglandins used. Only prostaglandin E_2, at the highest concentration used, 10^{-4} moles.l^{-1}, gave an increase significantly different from controls ($p < 0.05$).

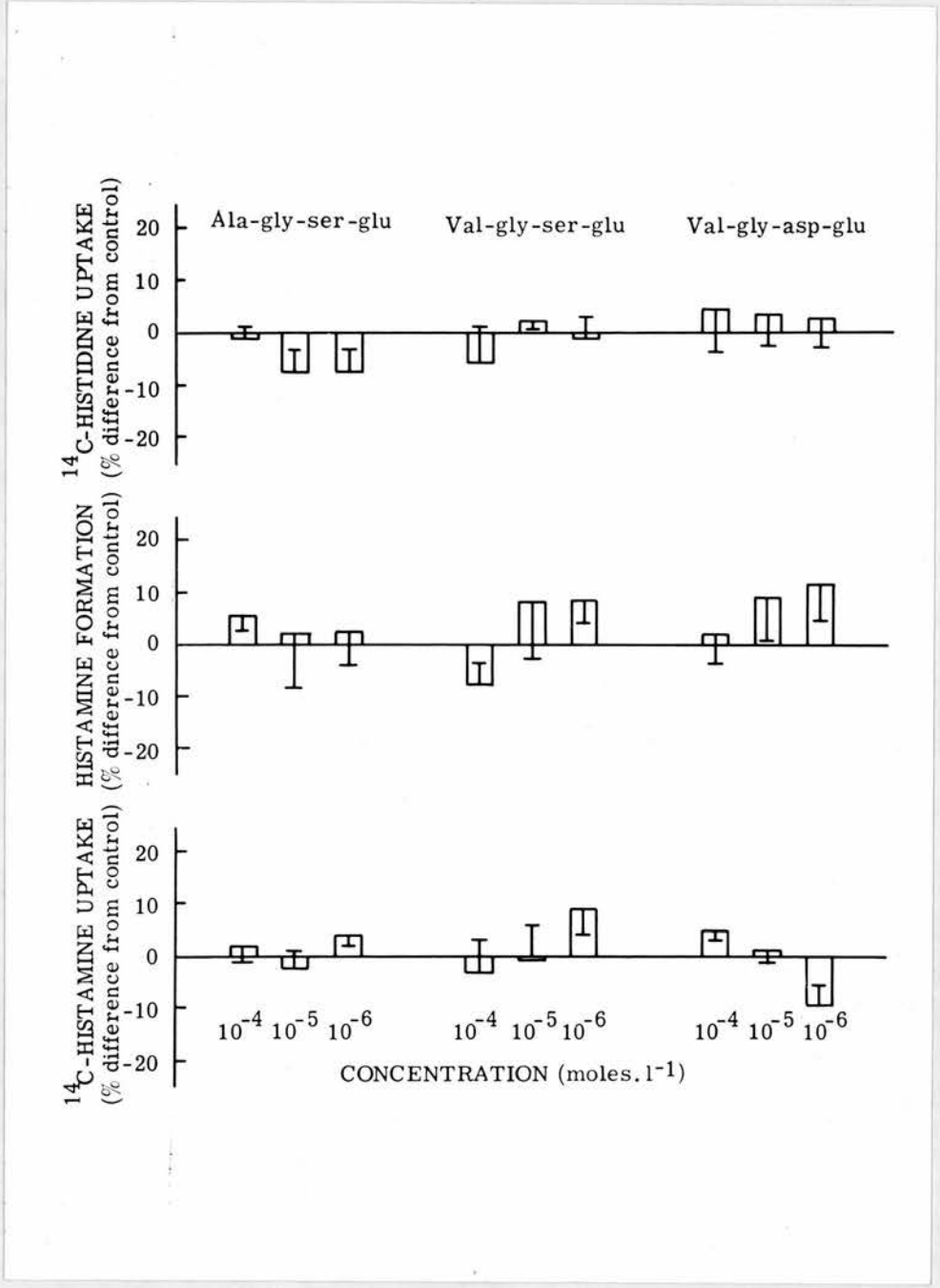

Fig. 32

The effect of the ECF-A tetrapeptides (Ala-Gly-Ser-Glu and Val-Gly-Ser-Glu) and a synthetic analogue (Val-Gly-Asp-Glu) on ^{14}C-histidine and ^{14}C-histamine uptake and ^{14}C-histamine formed. Each column represents the mean ± 1 S.E. of 3 experiments.

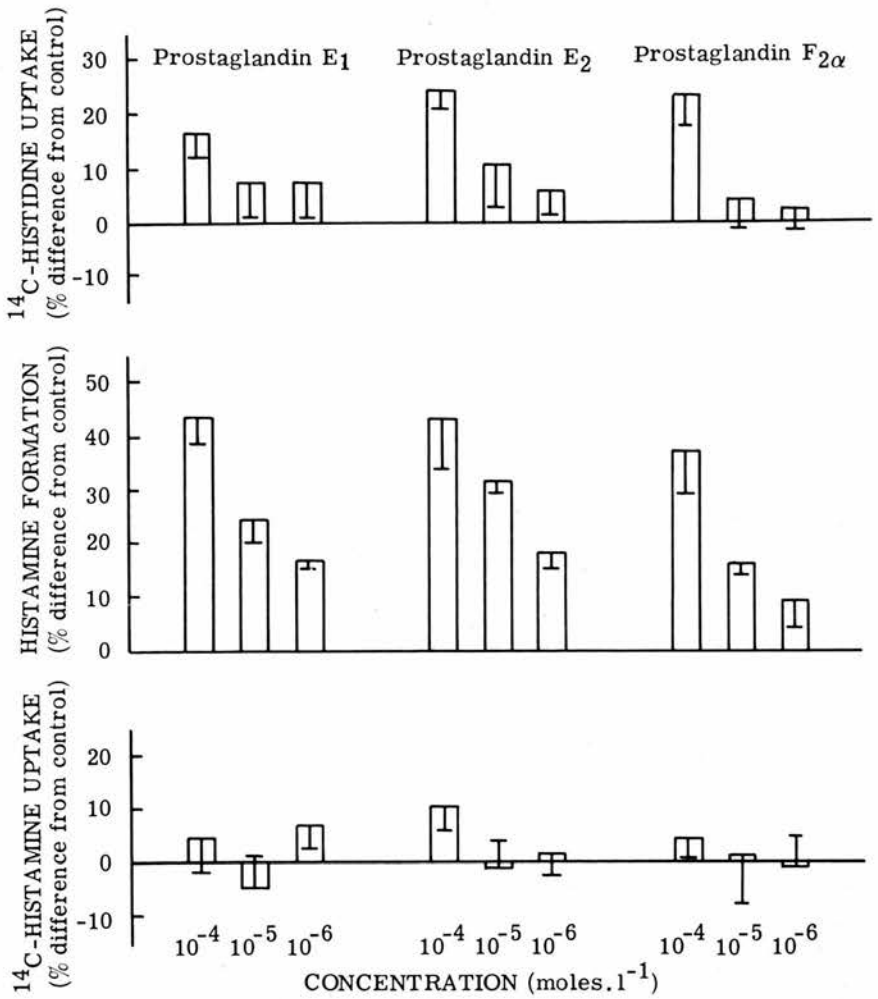

Fig. 33

The effect of prostaglandins (PGE_1, PGE_2 and $PGF_{2\alpha}$) on ^{14}C-histidine and ^{14}C-histamine uptake and ^{14}C-histamine formation. Each column represents the mean ± 1 S.E. of 3 experiments for ^{14}C-histidine uptake and ^{14}C-histamine formation and 5 experiments for ^{14}C-histamine uptake.

2.3 5-hydroxytryptamine and bradykinin

5-hydroxytryptamine had no significant effect on ^{14}C-histidine and ^{14}C-histamine uptake and ^{14}C-histamine formation at any of the concentrations used. Bradykinin did not significantly alter the amounts of ^{14}C-histidine and ^{14}C-histamine incorporated by the cell suspensions (Fig. 34).

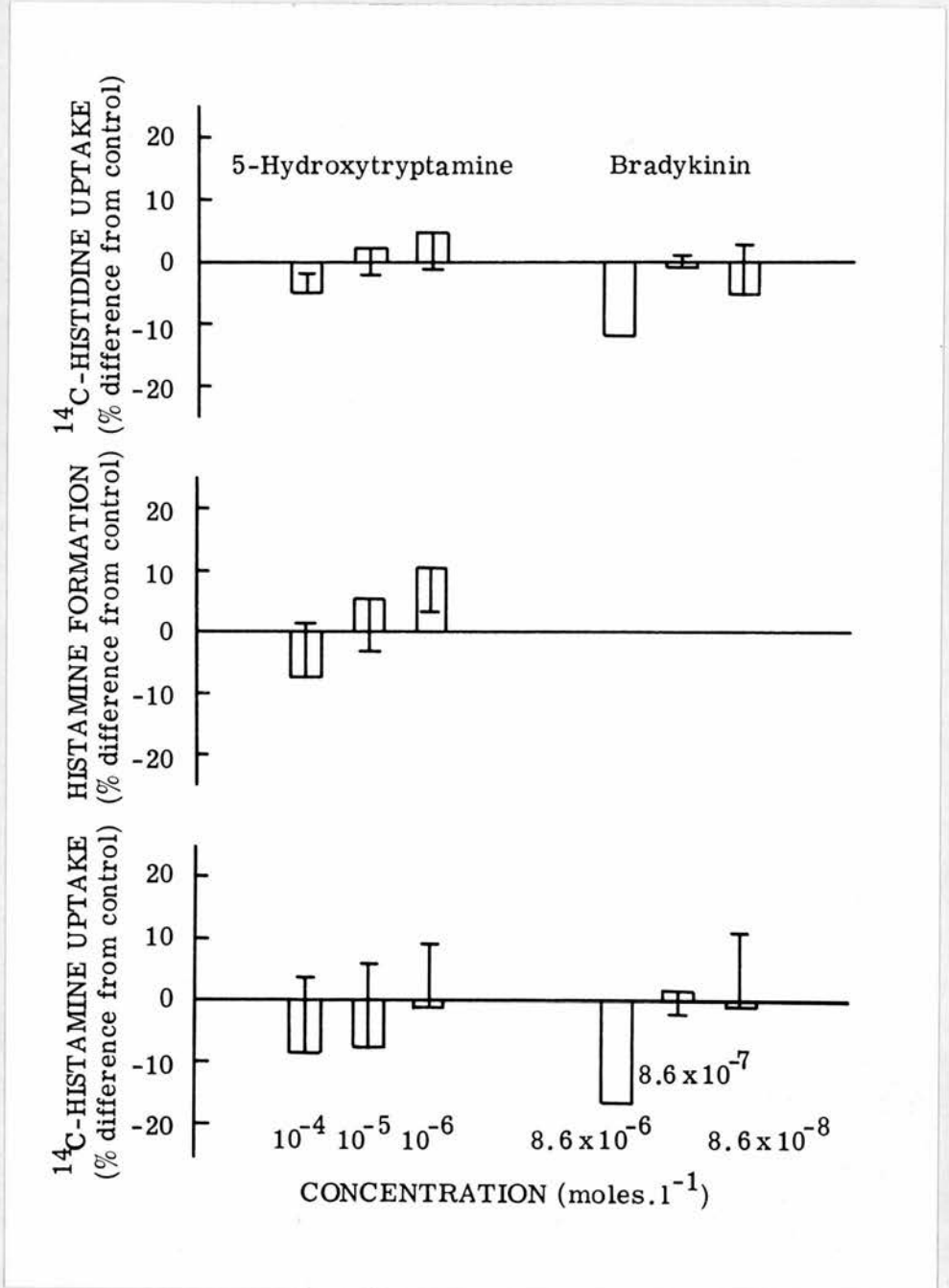

Fig. 34

The effect of 5-hydroxytryptamine on the uptake of ^{14}C-histidine and ^{14}C-histamine and ^{14}C-histamine formation and of bradykinin on ^{14}C-histidine and ^{14}C-histamine uptake. The results represent the mean ± 1 S.E. 5-hydroxytryptamine: ^{14}C-histidine uptake and ^{14}C-histamine formation - 4 experiments; ^{14}C-histamine uptake - 3 experiments. Bradykinin (8.6×10^{-7} and 8.6×10^{-8} moles.l^{-1}) - 2 experiments. The result for bradykinin at 8.6×10^{-6} moles.l^{-1} is for 1 experiment.

3.0 ANTI-INFLAMMATORY AND ANTI-ALLERGY AGENTS

The glucocorticoid, hydrocortisone, the non-steroidal anti-inflammatory drugs, indomethacin and aspirin, and the anti-allergy drug, Intal, had no significant effect on any of the parameters measured (Table XIII).

TABLE XIII

The effect of various drugs on the uptake of ^{14}C-histidine and ^{14}C-histamine and ^{14}C-histamine formation. The results represent the mean ± 1 S.E. or the mean of 2 • or the results of 1 ø experiment.

DRUG	CONCENTRATION (moles.l^{-1})	NO. OF EXPERIMENTS	^{14}C-HISTIDINE UPTAKE (% difference from control)	HISTAMINE FORMATION (% difference from control)	^{14}C-HISTAMINE UPTAKE (% difference from control)
Hydrocortisone	10^{-4}	3	-2.1 ± 4.5	-6.2	-2.1
	10^{-5}	2	-5.4	-15.3 ø	+7.4
	10^{-6}	3	+0.8 ± 3.7	-16.1	+6.6
Indomethacin	10^{-4}	3	+14.1 ± 7.8	+7.4 ± 7.1	-9.4
	10^{-6}	3	+15.8 ± 7.7	+6.6 ± 8.0	-18.8 ø
Aspirin	10^{-4}	3	+7.8 ± 8.0	+14.1 ± 12.0	-0.3
	10^{-6}	3	+4.1 ± 7.2	+16.3 ± 18.8	-18.1 ø
Disodium Cromoglycate	10^{-4}	2	+6.9	+18.0	-
	10^{-5}	2	+5.9	+5.2	-
	10^{-6}	2	+8.6	+1.2	-

TABLE XIII

4.0 SUMMARY

The ECF-A tetrapeptides, 5-hydroxytryptamine and bradykinin, did not significantly alter the uptake of ^{14}C-histidine and ^{14}C-histamine and ^{14}C-histamine formation.

The prostaglandins studied (PGE_1, PGE_2 and $PGF_{2\alpha}$) significantly increased the amount of ^{14}C-histamine formed. In addition, PGE_2 and $PGF_{2\alpha}$, also significantly increased the uptake of ^{14}C-histidine. The uptake of ^{14}C-histamine was unaffected by prostaglandins with the exception of the highest concentration of PGE_2.

None of the anti-inflammatory (hydrocortisone, aspirin, indomethacin) nor the anti-allergy (disodium cromoglycate) drugs used significantly altered the uptake of ^{14}C-histidine and ^{14}C-histamine and ^{14}C-histamine formation *de novo*.

SECTION V - THE EFFECT OF ANAPHYLACTIC HISTAMINE RELEASE AND THE PRESENCE OF VARIOUS CELL TYPES ON ^{14}C-HISTIDINE UPTAKE AND ^{14}C-HISTAMINE FORMATION

1.0 INTRODUCTION

The anaphylactic release of pharmacological mediators is a non-cytotoxic secretory process. Cells which have discharged their granule contents after an antigen-antibody interaction remain viable as judged by exclusion of vital dyes, electron microscopic morphology and by continued motility under the phase contrast microscope (see General Introduction). It seems reasonable to anticipate, therefore, that degranulated basophils are capable of repair and regranulation.

Eosinophils or their products have been frequently implicated as regulators of immediate type hypersensitivity (see Aims) and their possible role in tissue mast cell repair have been suggested. For this reason the interactions between eosinophils and basophils were investigated.

2.0 THE EFFECT OF HISTAMINE RELEASE

Incubation of sensitized basophils with the appropriate antigen caused the release of histamine. Histamine release, expressed as a percentage of the total histamine present in the cell suspensions prior to antigen challenge, after incubation with various dilutions of antigen is shown in Fig. 35.

The cells from guinea pigs injected with sheep's blood gave a dose-dependent release of histamine, when incubated with a blood cell lysate, which was significantly different from controls at a $^{1}/_{10}$, $^{1}/_{100}$ dilution ($p < 0.05$). The histamine release from the cells of an ovalbumin-stimulated animal challenged with purified ovalbumin showed a dose response below 4 $mg.ml^{-1}$. However, inhibition of release was observed at 5 $mg.ml^{-1}$. When sheep's blood and ovalbumin primed animals were challenged with purified ovalbumin and sheep's red cell lysate, respectively, the histamine release was the same as the control levels.

Guinea pigs treated with sheep's blood were used as the source of the sensitized basophils in experiments designed to investigate the effect of histamine release on the amounts of ^{14}C-histidine incorporated and ^{14}C-histamine formed *de novo*. Except for some preliminary experiments, the dilutions of red cell lysate used were $^{1}/_{20}$, $^{1}/_{100}$ and $^{1}/_{500}$ since with longer treatments and incubation times, cells challenged with undiluted lysate, i.e. final dilution $^{1}/_{10}$, tended to be less viable than unchallenged controls.

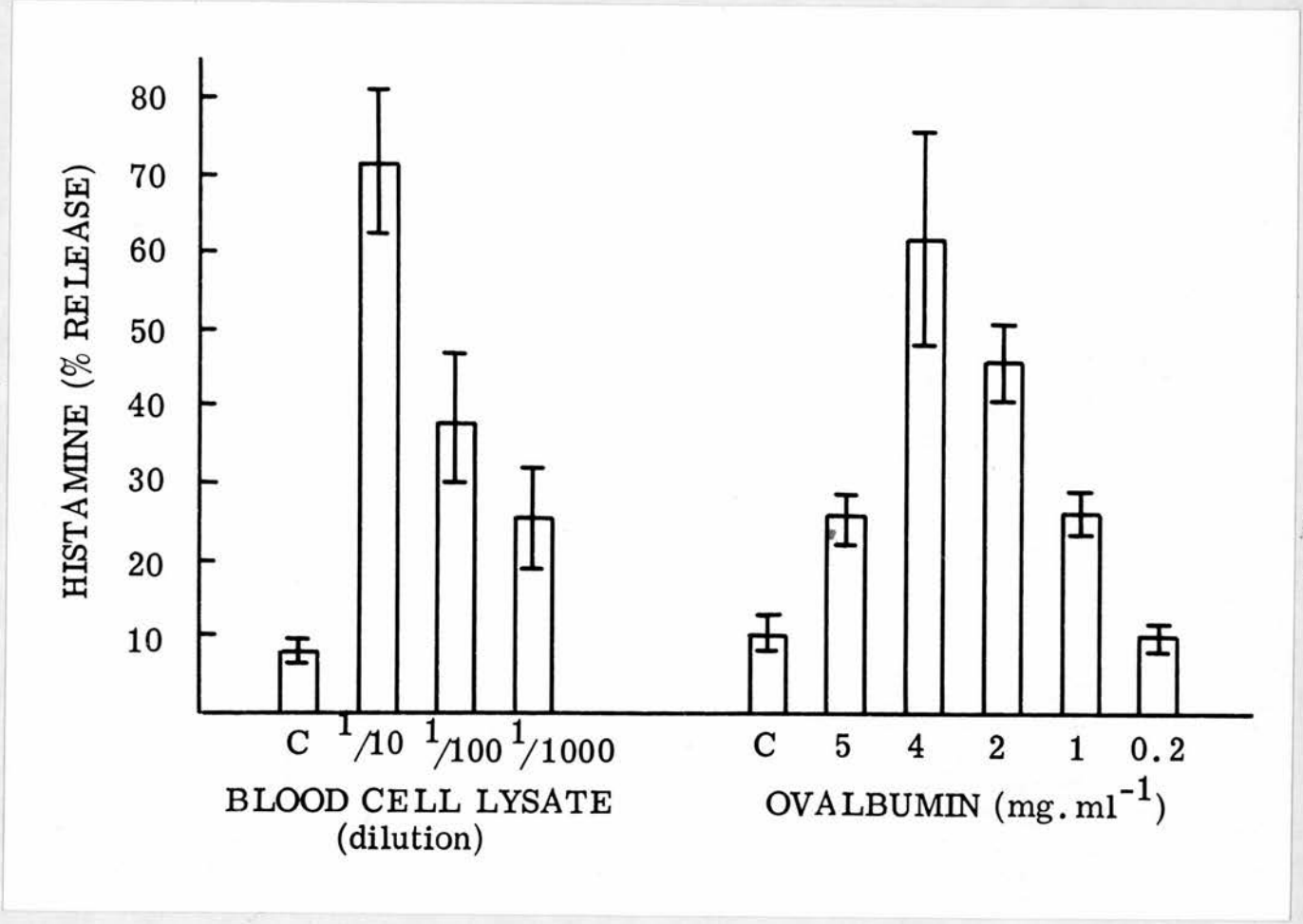

Fig. 35

The amount of histamine released by basophil-enriched cell suspensions from guinea pigs injected with sheep's blood or ovalbumin when incubated with the antigen used in their stimulation. Various dilutions of sheep's red cell lysate, or concentrations of ovalbumin were used. C = control (no antigen added).

Each column represents the mean ± 1 S.E. Sheep's blood primed animals - 4 experiments. Ovalbumin primed animals - 3 experiments.

2.1 ^{14}C-histidine uptake and ^{14}C-histamine formation immediately after antigen challenge

The percentage difference in ^{14}C-histidine uptake and ^{14}C-histamine formation, from unchallenged cells, after a 90 min incubation with ^{14}C-histidine immediately following challenge is shown in Fig. 36. There was a dose-dependent increase in the amount of ^{14}C-histidine incorporated and ^{14}C-histamine formed. The increase in the amount of ^{14}C-histamine synthesized was more marked than the increase in ^{14}C-histidine uptake. The amount of ^{14}C-histamine formed by basophils which had released a portion of their stored histamine was significantly greater than the amount of histamine formed by unchallenged controls at $^{1}/_{10}$ ($p < 0.05$) and $^{1}/_{1000}$ ($p < 0.001$) dilutions. Histamine release had no statistically significant effect on the amount of ^{14}C-histidine incorporated.

2.2 ^{14}C-histidine uptake and histamine formation 24 hr after antigen challenge

In another series of experiments challenged and control cells were cultured in Eagle's Minimal Essential Medium without histidine for 24 hr prior to incubation for 90 min with ^{14}C-histidine in Tyrode's buffer (Fig. 36). Under these conditions there was no significant difference in ^{14}C-histidine uptake between challenged and control cells. In contrast, the amount of ^{14}C-histamine synthesized from the ^{14}C-histidine incorporated was significantly increased following challenge with $^{1}/_{20}$ ($p < 0.05$) and $^{1}/_{500}$ ($p < 0.01$) dilutions of red cell lysate.

In one experiment the cells from the same guinea pigs were used both immediately after challenge and 24 hr later. The amount of ^{14}C-histidine incorporated and histamine

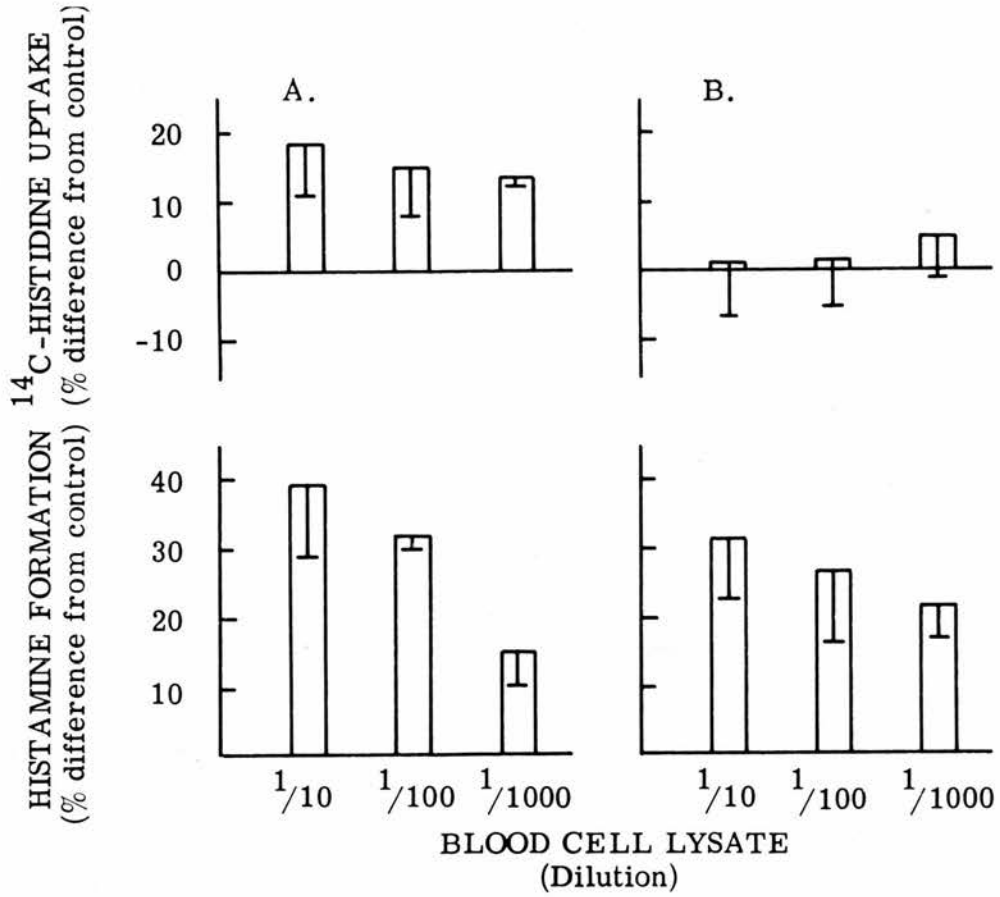

Fig. 36

The effect of anaphylactic histamine release induced by sheep's blood cell lysate on ^{14}C-histidine uptake and ^{14}C-histamine formation. A - immediately after challenge. B - after culture for 24 hr.

The release of histamine was dose-dependent in all experiments. Each column represents the mean ± 1 S.E. A - 1/10, 1/100 - 4 experiments; 1/1000 - 3 experiments. B - 6 experiments.

synthesized in the 90 min incubation with ^{14}C-histidine, by the same number of cells, was similar for both treatments.

2.3 Time course

In the two sets of experiments detailed above (parts 2.1 and 2.2) the cells were incubated with ^{14}C-histidine for 90 min. The results given in Fig. 37 show the amounts of ^{14}C-histidine incorporated and ^{14}C-histamine formed following prolonged incubations of up to 6 hr.

At incubation times above 30 min the antigen challenged cells incorporated more ^{14}C-histidine than unchallenged controls, the difference becoming more marked with time. The extent of the anaphylactic reaction did not influence the degree of enhancement of uptake since, while the various dilutions of antigen gave a dose-dependent release of histamine, they produced virtually identical ^{14}C-histidine incorporation.

A greater amount of ^{14}C-histamine was synthesized from the ^{14}C-histidine incorporated in the antigen-challenged cells at all time points. By 6 hr the cell suspension which was challenged with $^{1}/_{100}$ dilution of antigen formed over 80% more ^{14}C-histamine than the control, while the two other challenged preparations produced over 40% more.

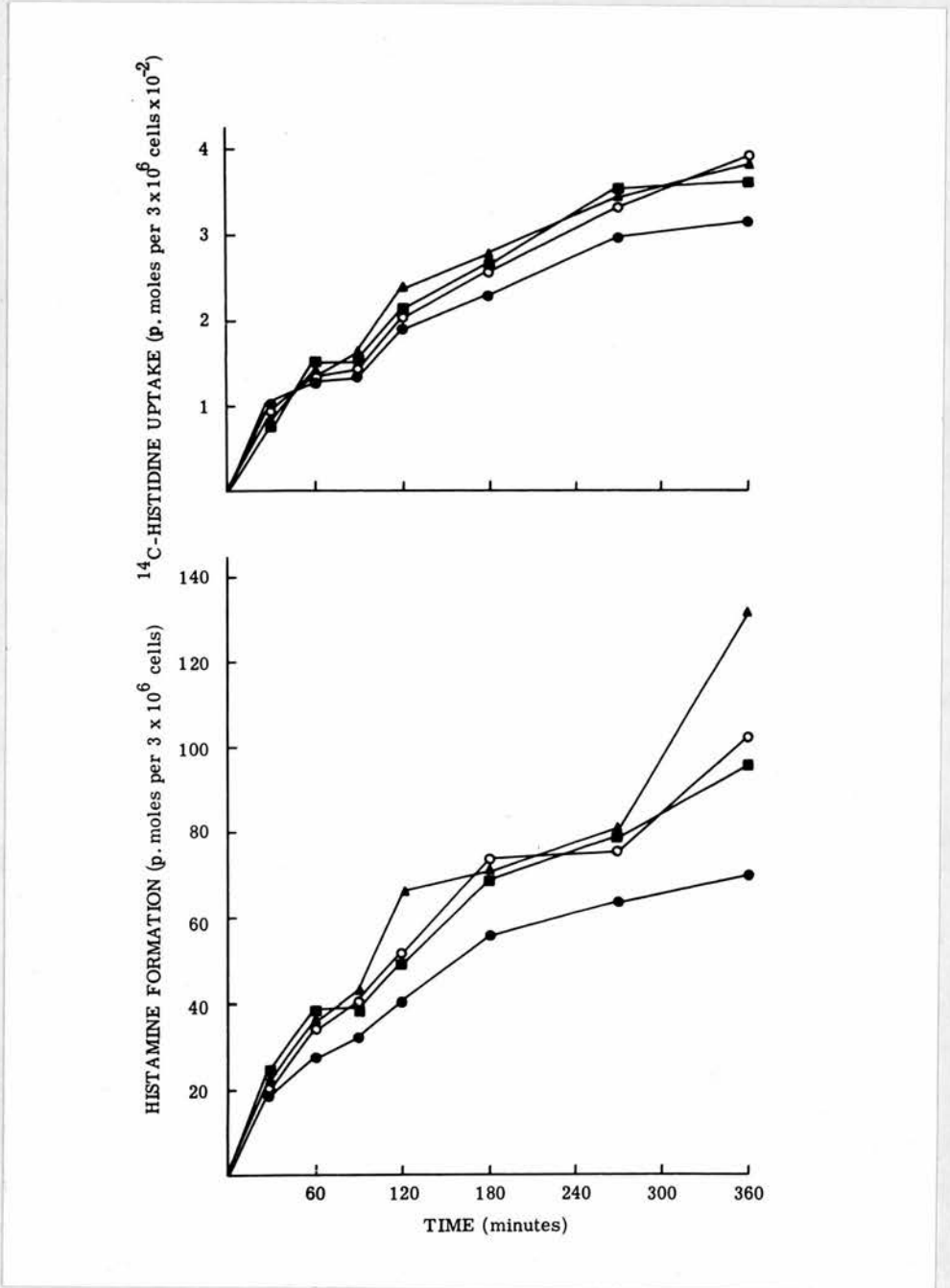

Fig. 37

Time course of ^{14}C-histidine uptake and ^{14}C-histamine formation after incubation with 1/20 (■ — ■), 1/100 (▲ — ▲) and 1/500 (O — O) dilution of sheep's blood cell lysate or control solution (● — ●). The results are from 1 experiment and the lysate caused a dose-dependent release of histamine.

3.0 EFFECT OF VARIOUS CELL TYPES

In Section I it was shown that basophil-enriched cell suspensions incorporated substantially more ^{14}C-histidine than the other cell types tested, viz. eosinophils, neutrophils, mononuclear cells and normal bone marrow cells. Further, ^{14}C-histamine formation de novo from the incorporated ^{14}C-histidine was measurable only in the basophil suspension.

The effect on ^{14}C-histidine uptake and ^{14}C-histamine formation of mixing large numbers of purified eosinophils, neutrophils and macrophages with basophil-enriched cell suspensions is shown in Figs. 38-43. Each figure shows the results from several experiments. It was impossible to pool the results from each experiment on the same cell type because of the variation in the number of cells used and the large variations in uptake encountered.

3.1 Eosinophils

Fig. 38 shows the results of three experiments where eosinophil-enriched cell suspensions (>90%) were mixed with basophil-enriched cell suspensions for 40 min prior to the addition of ^{14}C-histidine for 90 min. The ratios of eosinophils to basophils was 3:1 in the first experiment and 2:1 in the other two. The uptake of ^{14}C-histidine and the amount of ^{14}C-histamine formed by the mixture of cells was not significantly different from the amounts assimilated by the cells when incubated separately.

In other experiments suspensions rich in basophils and eosinophils and a mixture of the two cell types were cultured for 18 hr at 37^{o} in Eagle's Minimal Essential Medium containing 10% guinea pig serum. The cells were then

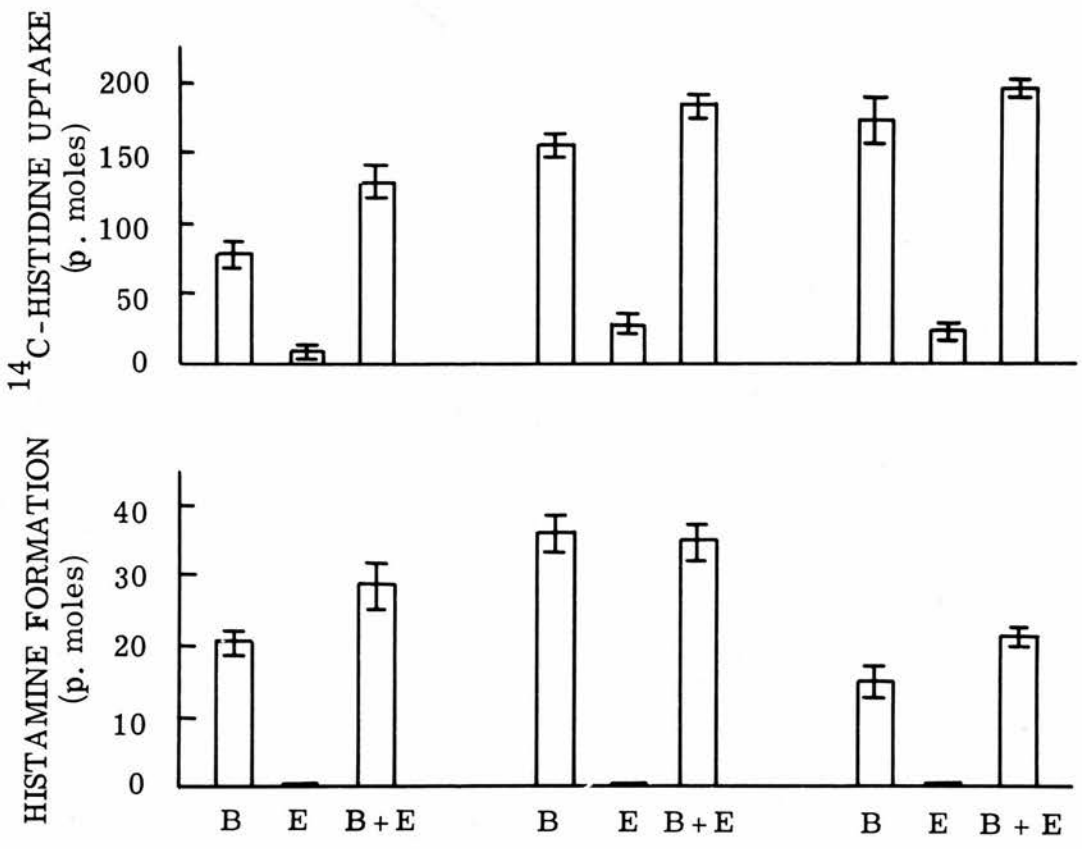

Fig. 38

The amount of ^{14}C-histidine taken up and ^{14}C-histamine formed by basophil-enriched cell suspensions (B), eosinophil-enriched cell suspensions (E) and a mixture of the two populations (B+E) during a 90 min incubation with ^{14}C-histidine, 40 min after mixing the cells. Each group of columns represent the results of 1 experiment (carried out in triplicate) and the bars ± 1 S.E.

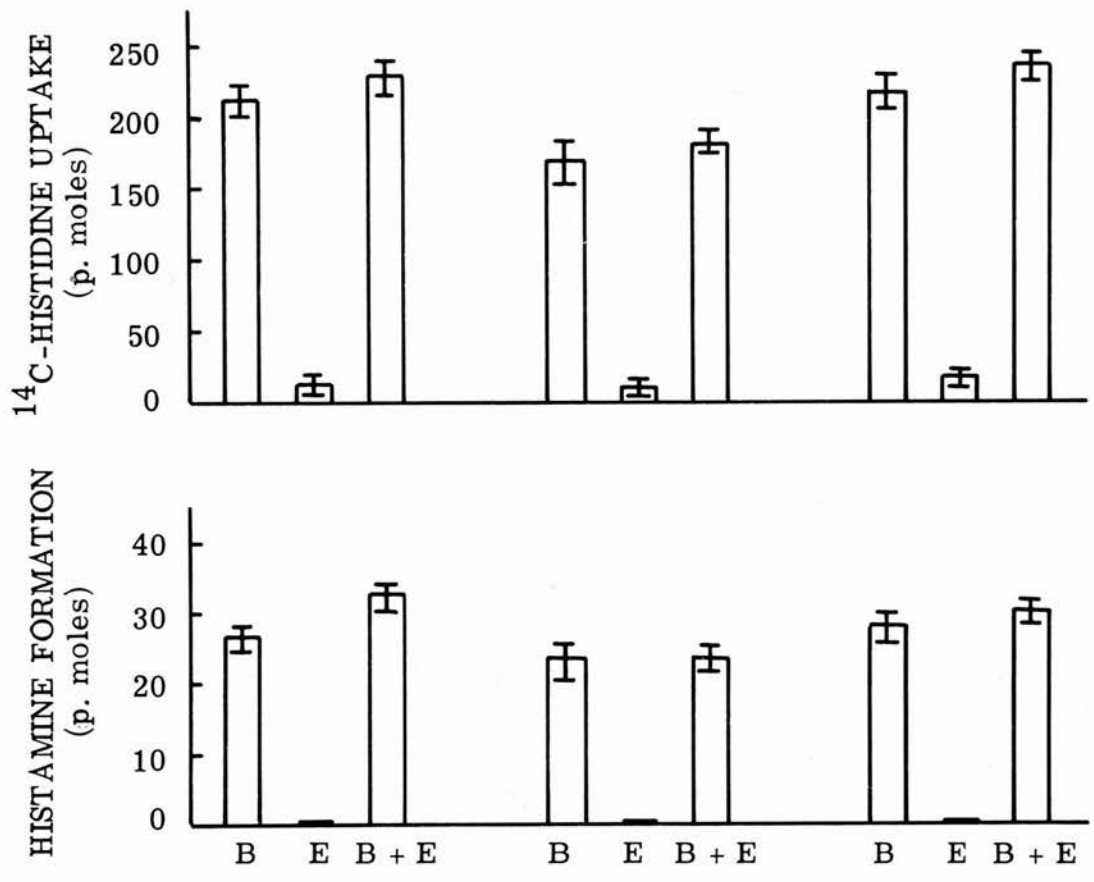

Fig. 39

The amount of ^{14}C-histidine taken up and ^{14}C-histamine formed by basophil-enriched cell suspensions (B), eosinophil-enriched cell suspensions (E) and a mixture of the two populations (B+E) during a 90 min incubation with ^{14}C-histidine 18 hr after mixing the cells. Each group of columns represent the results of 1 experiment (carried out in triplicate) and the bars ± 1 S.E.

incubated for 90 min in Tyrode's buffer in the presence of ^{14}C-histidine. The amounts of ^{14}C-histidine incorporated and ^{14}C-histamine formed in three different experiments are shown in Fig. 39. The ratios of eosinophils to basophils were 4:1, 5:1 and 4:1, respectively. Again there was no appreciable difference in ^{14}C-histidine uptake and ^{14}C-histamine synthesis between the mixture and the sum of the two cell types alone.

The eosinophil-enriched cell suspensions were obtained from the peritoneal cavity of guinea pigs stimulated by injections of horse serum. In the two experiments shown in Fig. 40 the eosinophils were incubated for 15 min with horse serum prior to mixing with the basophil-enriched cell suspensions and incubation with ^{14}C-histidine. Once more the total amount of ^{14}C-histidine incorporated and ^{14}C-histamine formed by the basophil and eosinophil suspensions alone was not significantly different from the amount accumulated by a mixture of the two cell types.

3.2 Neutrophils, macrophages and eosinophils

Neutrophil-rich (>95%), macrophage-rich (>90%) and eosinophil-rich (>86%) cell suspensions were added to cell suspensions rich in basophils in the ratio 4:1 for 40 min prior to the addition of ^{14}C-histidine. The concentration of histidine used in the three experiments shown in Fig. 41 was 21.8 μ.moles.l^{-1} (0.1 μCi:21.8 nmoles). None of the cell types tested produced any difference in the amount of ^{14}C-histidine incorporated or ^{14}C-histamine formed between the cell types and basophils alone or when mixed together.

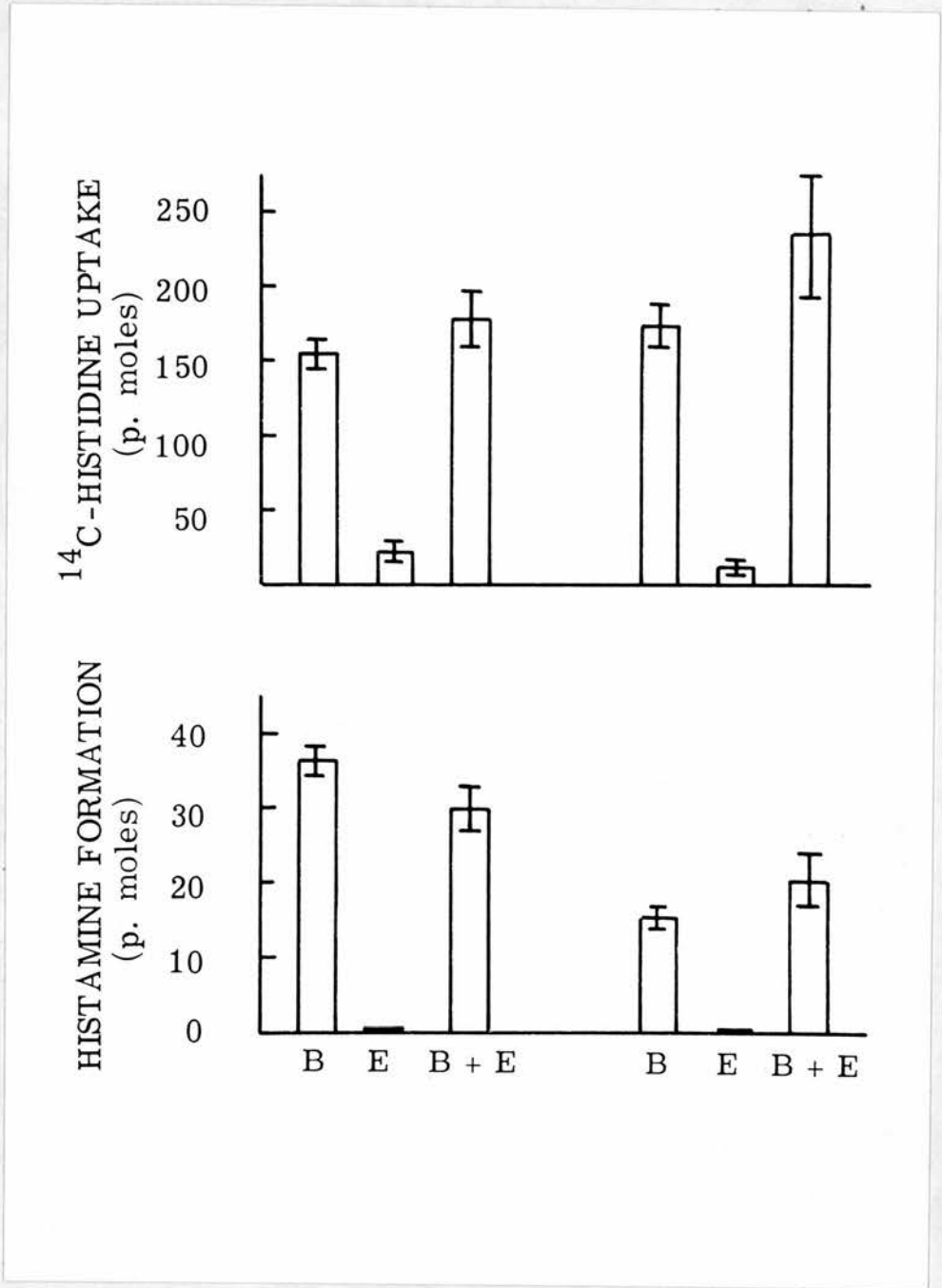

Fig. 40

The amount of ^{14}C-histidine taken up and ^{14}C-histamine formed by basophil-enriched cell suspensions (B), eosinophil-enriched cell suspensions incubated with horse serum (E) and a mixture of the two populations (B+E) during a 90 min incubation with ^{14}C-histidine, 40 min after mixing the cells. Each group of columns represent the results of 1 experiment (carried out in triplicate) and the bars ± 1 S.E.

4.0 THE EFFECT OF EOSINOPHILS ON ^{14}C-HISTIDINE UPTAKE AND HISTAMINE FORMATION BY BASOPHILS AFTER ANTIGEN CHALLENGE

Sensitized basophils, which had been challenged with antigen to release a portion of their stored histamine, were capable of incorporating a greater amount of ^{14}C-histidine and forming significantly more histamine than unchallenged basophils (part 2.1 - 2.3). If the slight uptake of ^{14}C-histidine by eosinophils is taken into account the presence of eosinophils at a ratio of 3:1 (Fig. 42) and 2:1 (Figs. 43 and 44) did not influence the enhanced uptake of ^{14}C-histidine or ^{14}C-histamine formation by the challenged basophils.

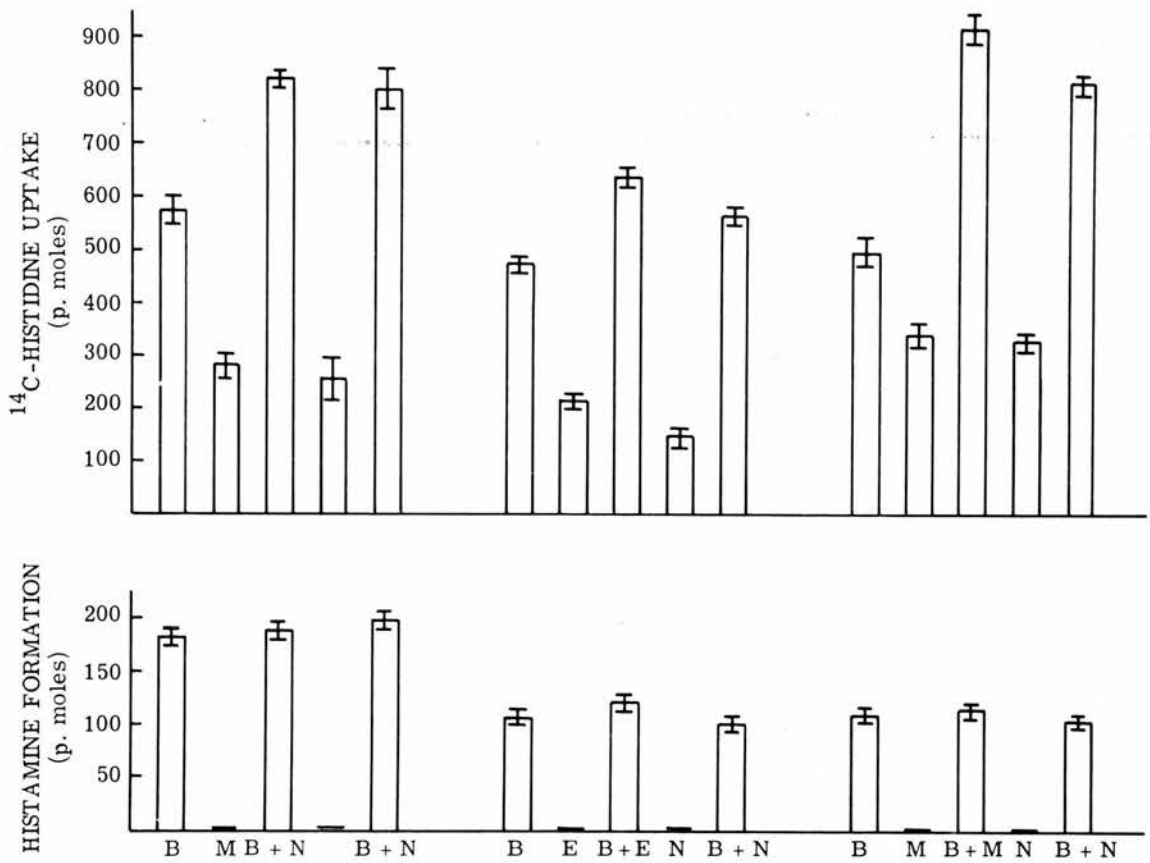

Fig. 41

The amount of ^{14}C-histidine taken up and ^{14}C-histamine formed by basophil (B), macrophage (M), neutrophil (N) and eosinophil (E) enriched cell suspensions, mixtures of basophils and macrophages (B+M), basophils and neutrophils (B+N) and basophils and eosinophils (B+E). Each group of columns represent the results of 1 experiment (carried out in triplicate) and the bars ± 1 S.E.

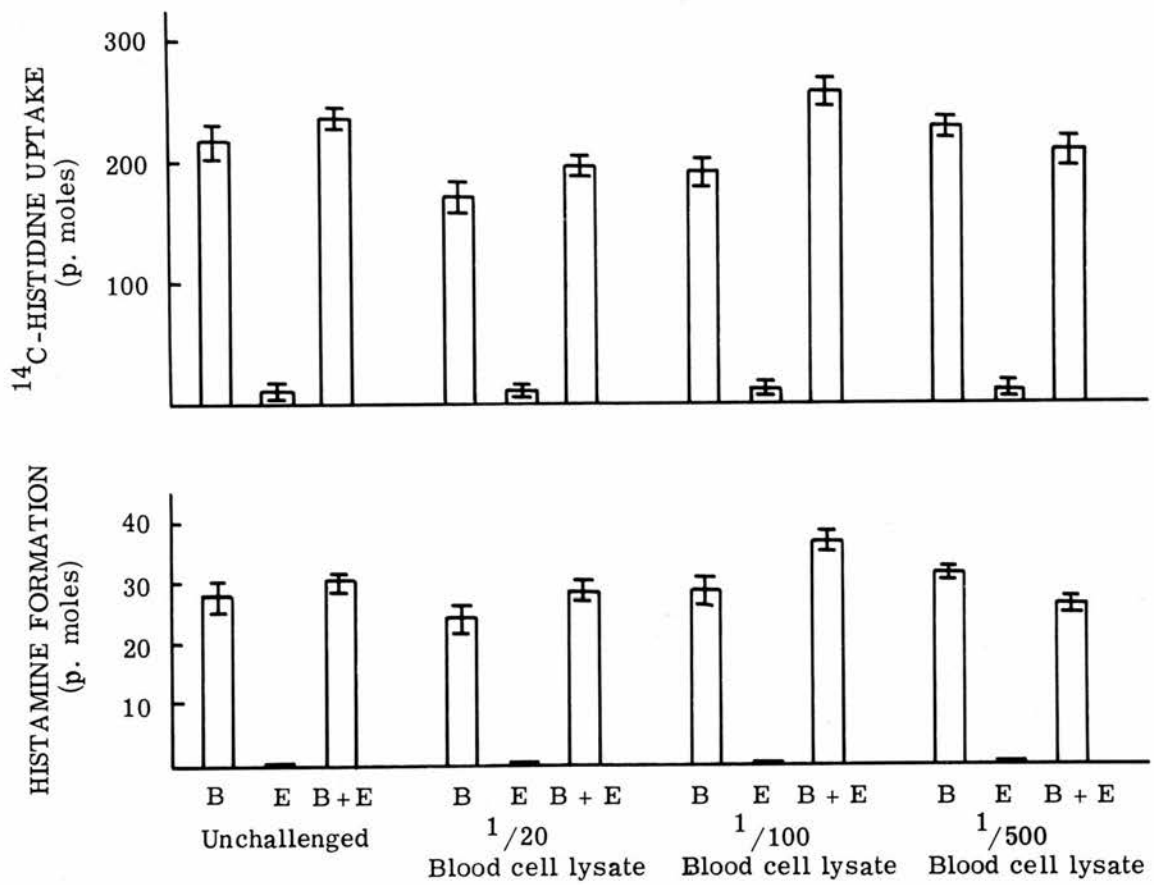

Fig. 42

The amount of ^{14}C-histidine taken up and ^{14}C-histamine formed by basophil-enriched cell suspensions (B), eosinophil-enriched cell suspensions (E) and a mixture of the two cell populations (B+E), all of which had been incubated with various dilutions of sheep's blood lysate or control. Each column represents the mean of 1 experiment (carried out in triplicate) ± 1 S.E.

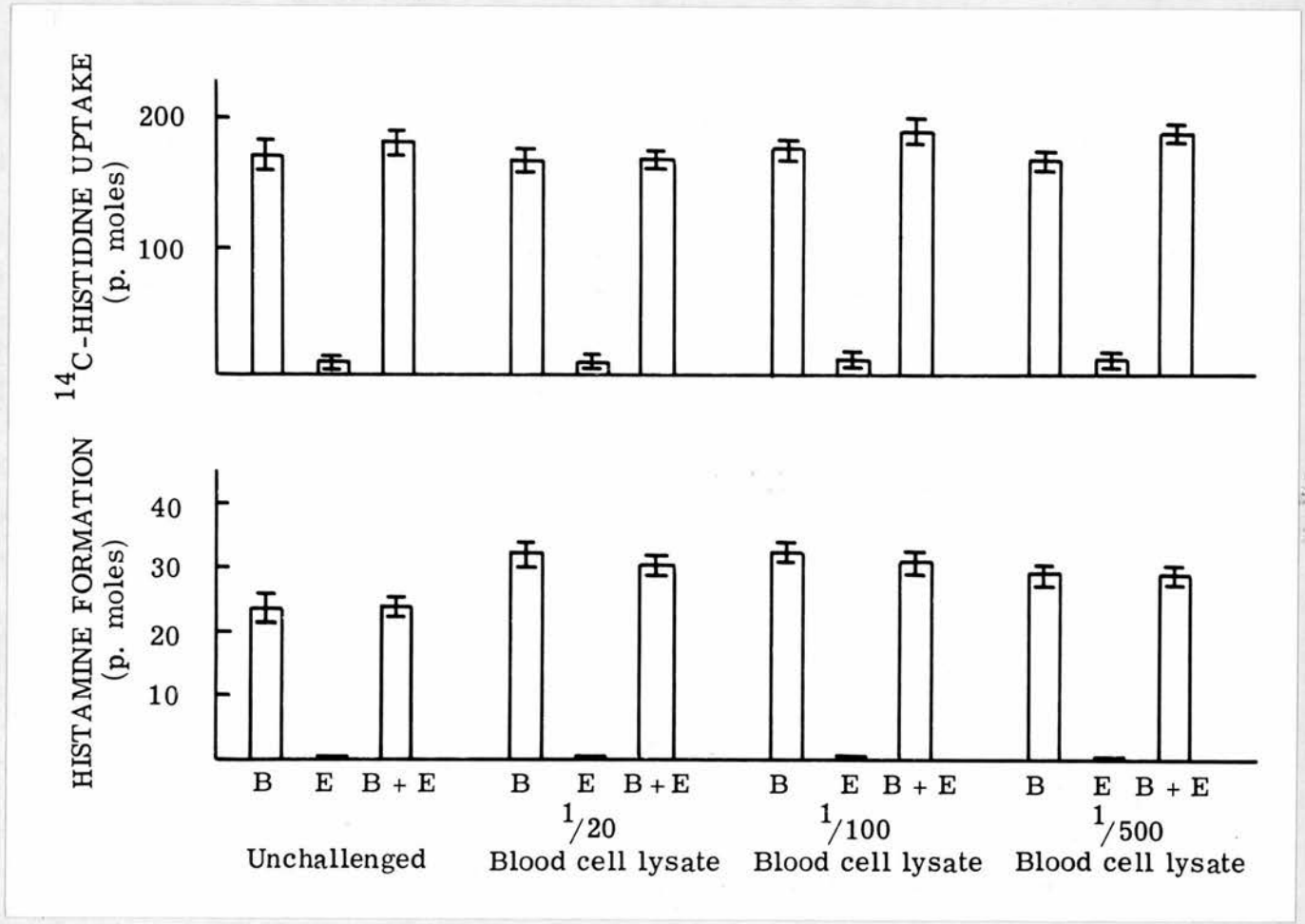

Fig. 43

The amount of ^{14}C-histidine taken up and ^{14}C-histamine formed by basophil-enriched cell suspensions (B), eosinophil-enriched cell suspensions (E) and a mixture of the two cell populations (B+E), all of which had been incubated with various dilutions of sheep's blood lysate or control. Each column represents the mean of 1 experiment (carried out in triplicate) ± 1 S.E.

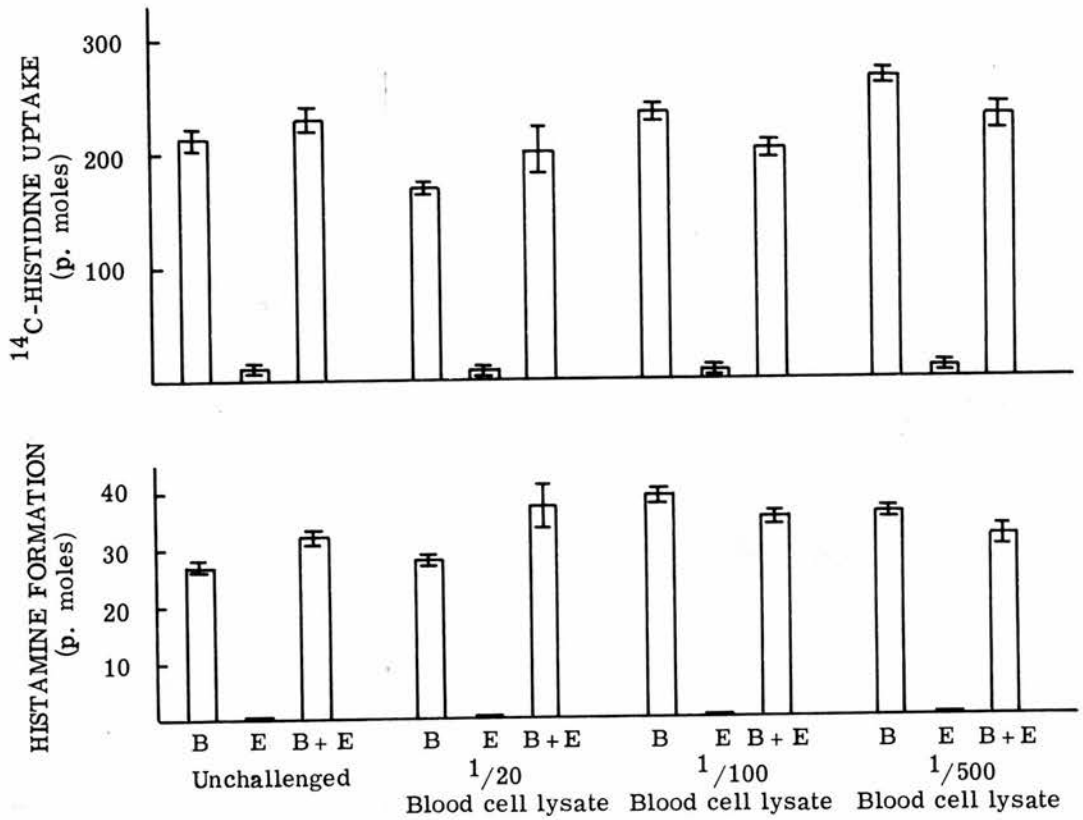

Fig. 44

The amount of ^{14}C-histidine taken up and ^{14}C-histamine formed by basophil-enriched cell suspensions (B), eosinophil-enriched cell suspensions (E) and a mixture of the two cell populations (B+E), all of which had been incubated with various dilutions of sheep's blood lysate or control. Each column represents the mean of 1 experiment (carried out in triplicate) ± 1 S.E.

5.0 EFFECT OF EOSINOPHIL-DERIVED PRODUCTS

Purified human eosinophil cationic protein and lysozyme had no significant effect on the amount of ^{14}C-histidine incorporated or ^{14}C-histamine formed (Fig. 45). A crude preparation from the granules of guinea pig eosinophils was also without effect under the conditions used.

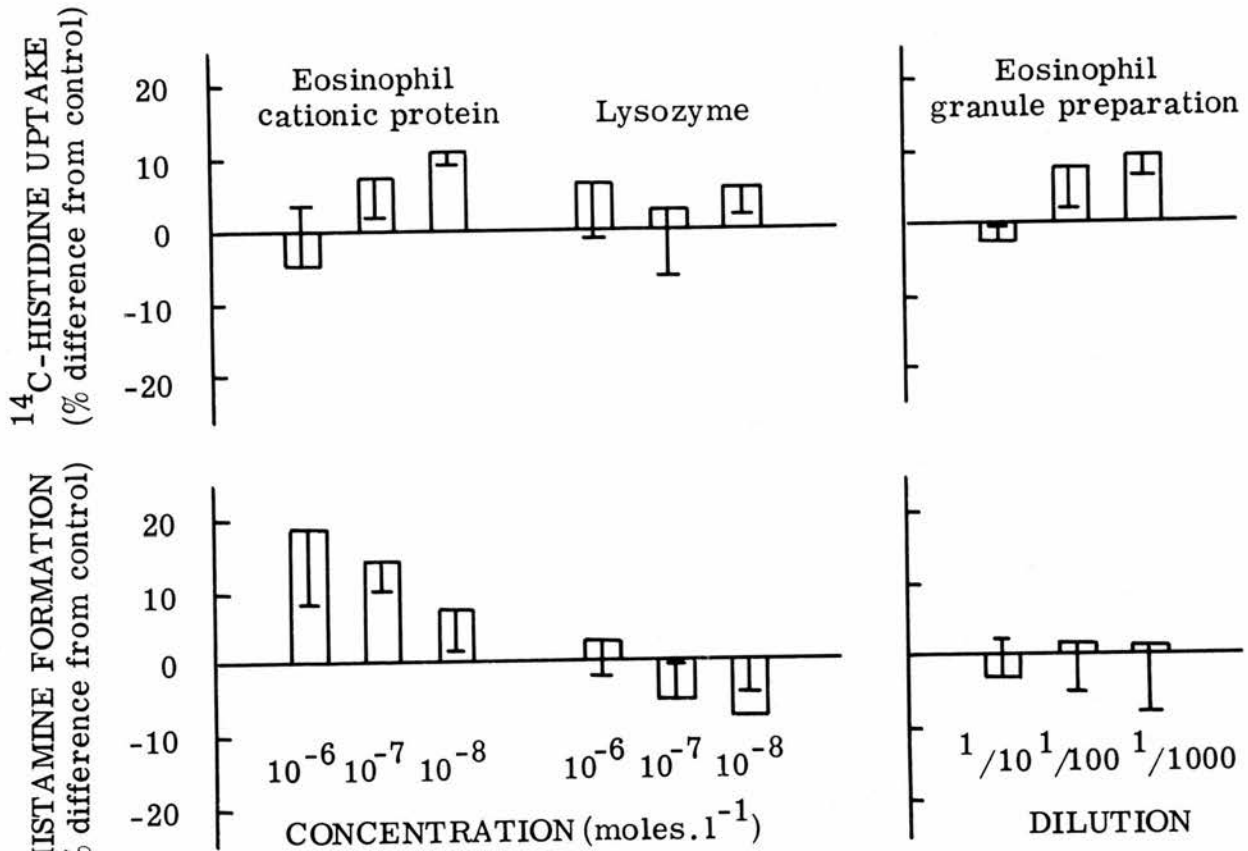

Fig. 45

The effect of eosinophil derived products (human eosinophil cationic protein, lysozyme and a guinea pig eosinophil granule preparation) on ^{14}C-histidine uptake and ^{14}C-histamine formation. Each column represents the mean ± 1 S.E. of 3 experiments.

6.0 RELEASE OF ^{14}C-HISTAMINE AND ^{14}C-HISTIDINE

Basophil-enriched cell suspensions, from guinea pigs primed with sheep's blood, gave a dose-dependent release of ^{14}C-histamine when challenged with a sheep red cell lysate after incubation with ^{14}C-histidine (Fig. 46). The ^{14}C-histamine released was calculated as a percentage of the total ^{14}C-histamine present after spontaneous release had been subtracted. All the antigen dilutions used gave a release which was significantly different from control ($p < 0.001$).

^{14}C-histidine was also released, in a dose-dependent fashion, by antigen from cell suspensions which had been incubated with ^{14}C-histidine in the presence or absence of NSD 1055 (Fig. 47). The release is expressed as a percentage difference from unchallenged controls since the basophil suspensions were not 100% pure and a portion of the total ^{14}C-histidine present before challenge would be associated with the contaminating cells and, therefore, not available for antigen-induced release.

There was a significant release of ^{14}C-histidine from the cells incubated with ^{14}C-histidine by all dilutions of antigen used, $^{1}/_{10}$ ($p < 0.05$) and $^{1}/_{100}$ and $^{1}/_{1000}$ ($p < 0.01$). When the cells were incubated with ^{14}C-histidine in the presence of the histidine decarboxylase inhibitor, NSD 1055, to prevent the conversion of ^{14}C-histidine to ^{14}C-histamine, there was also a dose-dependent release of radioisotope. In the presence of NSD 1055 the release of ^{14}C-histidine was significantly different from controls at antigen dilutions of $^{1}/_{10}$ and $^{1}/_{1000}$ ($p < 0.05$) and $^{1}/_{100}$ ($p < 0.01$).

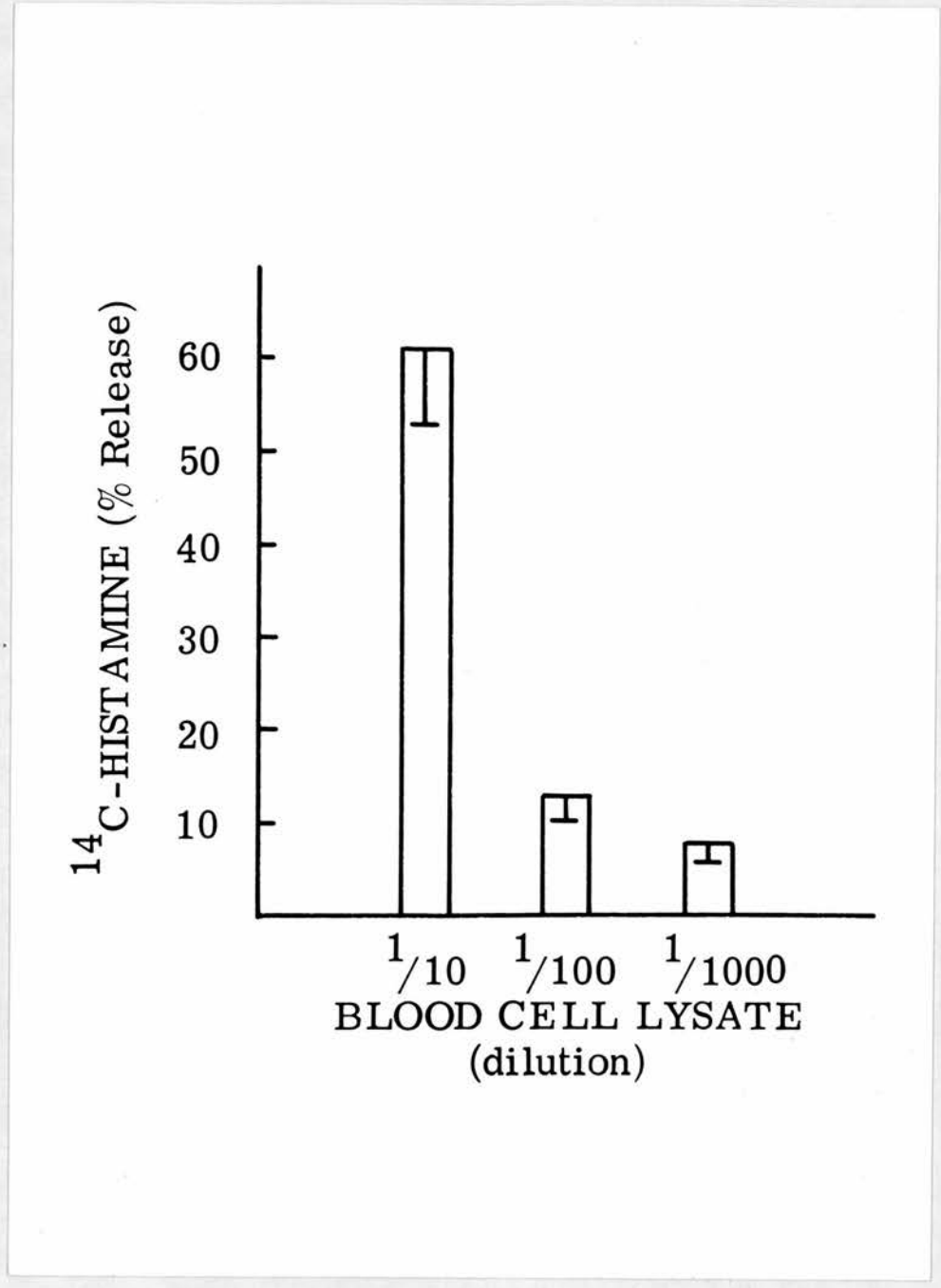

Fig. 46

The amount of ^{14}C-histamine released by various dilutions of antigen (sheep's blood cell lysate) from basophil-enriched cell suspensions incubated with ^{14}C-histidine. Each column represents the mean ± 1 S.E. of 7 experiments.

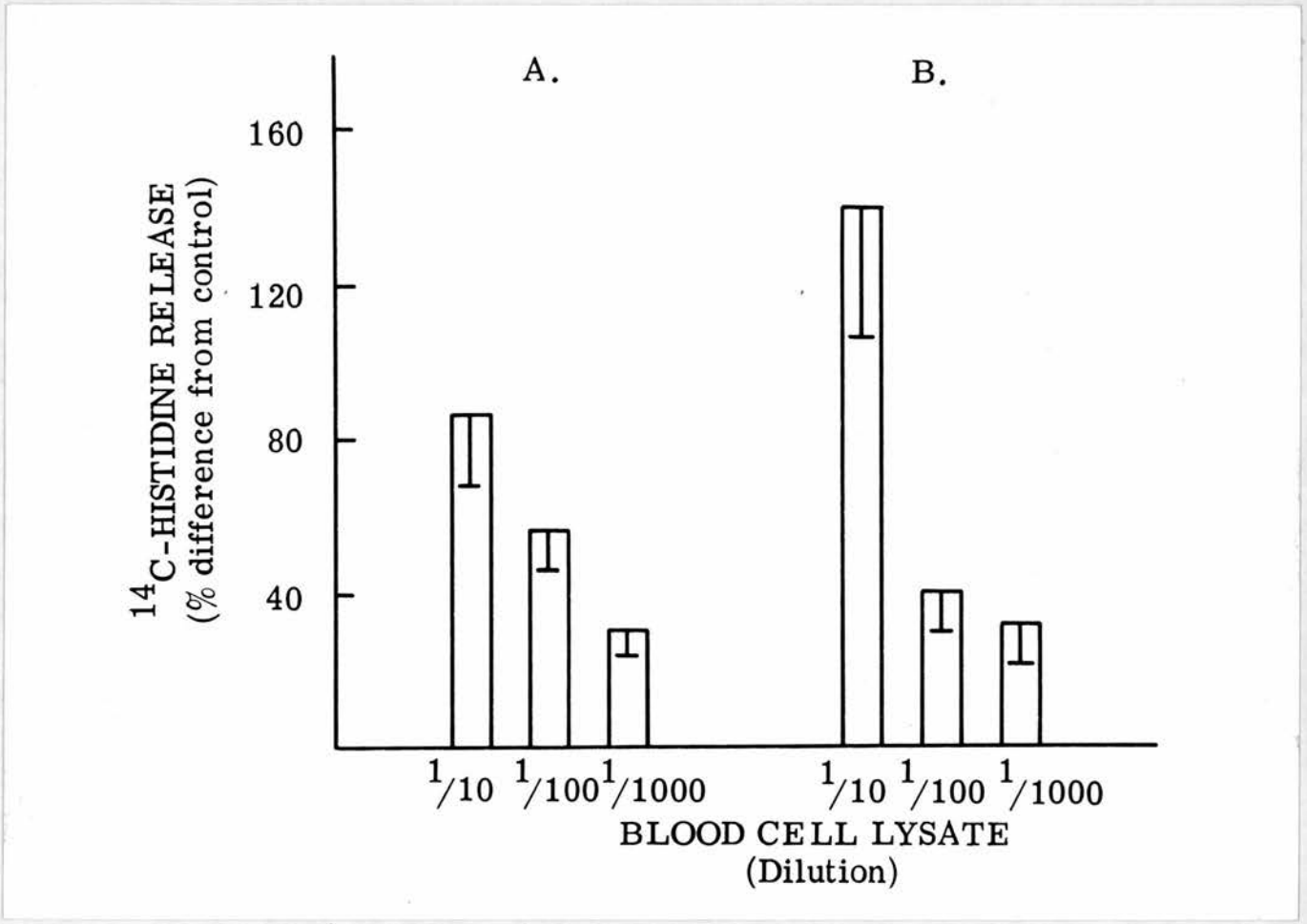

Fig. 47

The amount of ^{14}C-histidine released by various dilutions of antigen (sheep's blood cell lysate) from basophil-enriched cell suspensions incubated in the absence (A) or presence (B) of NSD 1055 (10^{-5} moles.1^{-1}). Each column represents the mean ± 1 S.E. of 6 experiments.

7.0 SUMMARY

Basophils from guinea pigs sensitized with sheep's blood or ovalbumin released histamine following challenge with specific antigen. The amounts of histamine released were related to the concentrate of antigen used.

Basophils which had undergone an "anaphylactic" reaction were still capable of incorporating ^{14}C-histidine and forming ^{14}C-histamine *de novo*. Indeed, there was a time-dependent increase in ^{14}C-histidine uptake and ^{14}C-histamine formation by cells depleted of histamine by antigen challenge when compared to unchallenged controls.

Eosinophils, neutrophils and macrophages under a variety of conditions had no effect on ^{14}C-histidine incorporation or ^{14}C-histamine synthesis by "unchallenged" basophils and in the case of eosinophils, on "challenged" cells.

The uptake of ^{14}C-histidine and histamine formation *de novo* was unaffected by purified eosinophil cationic protein.

Basophils which had been incubated with ^{14}C-histidine gave a dose-dependent release of ^{14}C-histamine when challenged with antigen. Under the same conditions a proportion of the incorporated ^{14}C-histidine was also released.

CHAPTER V - DISCUSSION

SECTION I - CHARACTERIZATION OF THE UPTAKE OF HISTIDINE AND HISTAMINE AND HISTAMINE FORMATION

The method developed by Dvorak et al (1974) for the preparation of basophil-rich suspensions has been found to be reliable and reproducible. In the present study the most important single factor which determined the final purity was the initial basophil content of the bone marrow preparations. The bone marrow from sheep's blood treated guinea pigs contained from 1.6 to 19.4% (mean 7.9) basophils, and that of ovalbumin treated animals 1.2 to 10.4% (mean 5.0). The overall purification achieved was four- to five-fold and, therefore, since at least 20% basophils were required in the final suspension, bone marrow containing less than 5% basophils was unsuitable for routine experiments and rejected.

Sheep's blood proved to be the most reliable antigen(s) and was used for virtually all the experiments. The degree of purification achieved was in agreement with the values originally reported by Dvorak et al (1974). However, the same number of nucleated cells could be obtained from two animals, whereas five, of similar weight, were required by the original workers.

The translocation of solutes across a cell membrane may proceed by three different routes: non-mediated diffusion, active mediated transport and passive mediated transport.

In non-mediated flux, or passive diffusion, the solute molecules remain in their initial molecular species throughout the transport process. Non-mediated transport is at all times dependent on the concentration of the solute and is entirely the result of simple physical diffusion down a concentration or electrochemical gradient.

Mediated transport exhibits saturation kinetics, suggestive of specific sites to which the substance undergoes reversible

binding. A second characteristic of mediated transport is specificity for the material transported. Such transport systems may also be inhibited quite specifically by structurally related substances or non-competitively by reagents capable of blocking or altering functional groups of proteins. There are two types of mediated transport processes, active and passive.

An active mediated process, which carries a substance against a chemical or electrochemical gradient, is dependent upon a source of metabolic energy and is unidirectional (Ussing, 1949; Danielli, 1954). Passive mediated transport, sometimes called facilitated diffusion, requires no input of free energy other than that needed to maintain the structure of the cell membrane and may be in either direction depending on the relative concentrations in the two compartments (Stein, 1967).

The uptake of ^{14}C-histidine by guinea pig bone marrow basophils fulfilled most of the characteristics of active mediated transport. Thus it exhibited saturation kinetics (Figs. 3A and 9A and Table VII), was inhibited by several other amino acids (Figs. 11-14) but was not affected by the D-stereoisomer (Fig. 10) and was dependent on the integrity of a number of metabolic pathways (Figs. 16-18).

The uptake of ^{14}C-histamine was non-saturable under the conditions used (Fig. 3A) but it was inhibited by several of its major catabolites (Fig. 24). ^{14}C-histamine uptake was virtually independent of metabolic energy (Table XI) but required the presence of sodium and an active sodium pump (Fig. 19). These facts suggest that histamine enters the cells studied by passive mediated transport which requires the movement of sodium to compensate for its net positive charge at a neutral pH.

Between 20 and 30% of the ^{14}C-histidine taken up by the basophil-enriched cell suspensions was converted to ^{14}C-histamine whereas in eosinophil-rich, neutrophil-rich, macrophage-rich and normal bone marrow cell suspensions there was no detectable ^{14}C-histamine formation. The enzyme responsible for the formation of histamine, L-histidine decarboxylase, is controlled by a complex interaction of various mechanisms (see Introduction, Section I, part 5.1). The activity varies with substrate concentrate which affects the pH optimum of the enzyme. Histidine and histamine may, in certain circumstances, form cyclic compounds with the co-enzyme, pyridoxal 5'-phosphate. In this feedback mechanism newly formed histamine could inhibit its own synthesis; excess histidine could also have the same effect on amine formation.

At a comparatively low extracellular histidine concentration, 70 μ moles.l^{-1}, the amount of histamine formed apparently reached a constant maximal level. This was probably due to the saturation of the intracellular histidine decarboxylase under the conditions used. However, at concentrations above 100 μ moles.l^{-1} a substantial amount of unlabelled histidine was present and this lowered its specific activity to such an extent that accurate determination of histamine formation was impossible by the chromatographic method used. Therefore, the amount of histamine formed at high concentrations could not be determined.

The uptake of histidine by bone marrow cell suspensions rich in basophils, as measured in this study, was the summation of several processes. The value obtained was a measure of the amount of histidine extracted from the medium by the cell

suspension and included: histidine which was in the process of passing through the membrane or may have been bound to the membrane itself; some which had passed through the membrane and was still essentially free histidine; a portion which had been decarboxylated to histamine and bound in the granules; and that which had been incorporated into proteins. The uptake of ^{14}C-histidine could also have involved the leakage of ^{14}C-histidine and newly formed ^{14}C-histamine from the cells during the incubation and washings. However, insignificant amounts of labelled histidine and histamine were detected in the supernatant of cell suspensions which were the controls for the experiments on anaphylactic histidine and histamine release (Results, Section V, part 6.0). These cells had been incubated with ^{14}C-histidine, washed and then incubated at 37^{o} for 15 min. The amount of spontaneously released radioisotope was less than 3% of that present within the cells.

The ^{14}C-histamine uptake was a measure of the histamine which was associated with the cells. Its precise location was not defined but it may have been bound to the cell membrane, have passed through into the cell or have been bound in the granules. There was no evidence that the histamine was metabolized within the cells since the chromatographic profile of the incorporated histamine extracted by multiple freeze-thawing was similar to that of the histamine used in the incubation.

The cell suspensions used for the study of histidine and histamine uptake contained, on average, 30% basophils. Therefore, a certain proportion of the incorporated radio-isotope was associated with other cell types. However, the

amount of histamine formed was a direct measure of the synthesis of histamine by the basophils since no other cell type tested was able to decarboxylate histidine. Histamine formation was, therefore, a gauge of variations of histamine metabolism in basophils.

The evidence for the basophil being more efficient in incorporating histidine and forming new histamine than other leucocytes depended on these activities (i) being superimposed with the basophil peak following cell separation (Fig. 6); (ii) correlating with the percentage of basophils in various cell suspensions (Fig. 7); and (iii) being significantly higher in purified basophil preparations when compared to preparations of other cell types (Fig. 8). All these conditions were fulfilled by the data presented.

Although bone marrow basophils were used in all the experiments, cells isolated from the peripheral blood gave similar results in terms of ^{14}C-histidine and ^{14}C-histamine uptake and ^{14}C-histamine formation (Table III). Blood cells incorporated about half the amount of histidine taken up by bone marrow derived cells. Although less histamine *in toto* was formed by the blood cells, the percentage of the incorporated histidine which was converted to histamine was about the same in the cells from the two sources (blood cells converted 26.1% and bone marrow cells 23.9%).

This may have been a reflection on the maturity of the cells involved. The less mature bone marrow cells take up more histidine requiring a large proportion for cellular processes other than histamine formation, e.g. protein synthesis. On the other hand, the fully differentiated

blood cells may have partially lost the ability to incorporate histidine, and possibly other amino acids, since they do not require these as precursors for protein synthesis to the same extent as bone marrow cells. However, the basophils from the two sources are capable of decarboxylating histidine, albeit to different extents.

When blood and bone marrow derived cells were incubated with ^{14}C-histamine under similar conditions there was no significant difference in the amounts of histamine incorporated.

Using similar chromatographic assays but different incubation procedures Galli et al (1976) showed that guinea pig blood basophils could synthesize ^{3}H-histamine from ^{3}H-histidine and that the uptake of exogenous ^{3}H-histamine was relatively trivial. These workers also demonstrated that histamine synthesis by lymphocyte cultures was essentially undetectable. Ten times as much new histamine was formed in the first hour of incubation with ^{14}C-histidine in the bone marrow cell suspensions than reported by Galli for cells from peripheral blood. However, after 6 hr the results were essentially in agreement. The initial variations are probably explained by the differences in the incubation conditions. Galli et al (1976) used a tissue culture medium while in the present study, which was designed primarily to investigate histidine uptake over short periods, a simple balanced salt solution was used since the uptake of histidine was partially inhibited by the presence of certain amino acids.

The uptake of histidine and histamine and histamine synthesis has been studied in cells from tissues of various species (Furano and Green, 1964; Lindell et al, 1961; Schayer, 1956; Day and Green, 1962a and b). Cabut and

Haegermark (1966), using rat peritoneal mast cells, and Day and Stockbridge (1964), studying mouse neoplastic mast cells, suggested that preformed histamine was accumulated by passive diffusion and binding to the granules. Later, work by Heisler and Uvnas (1972) showed that passive diffusion of histamine was probably of minor significance and that transport was due to facilitated diffusion. Cabut and Haegermark (1968) produced evidence that histidine transport into rat peritoneal mast cells was an active mediated process, in agreement with the data here.

Rat mast cells incorporated substantially less histidine than the same number of basophils whereas histamine uptake and formation was not significantly different in the two cell types (Table IX). The greater histidine incorporation by basophils may have been due to the presence of immature, rapidly developing cells within the populations studied. This is emphasized when the amount of histamine formed is considered. The rat mast cells converted 83% of the incorporated histidine to histamine compared to only 31% for the basophils. It is, therefore, probable that a large portion of the histidine taken up by the basophils is used for other processes, e.g. protein synthesis. Alternatively, the rat mast cells may have lost part of their transport system for histidine and other amino acids as they matured and less amino acids were required for cell development. Mast cells at the same stage of development as the basophils may possess the same capacity for histidine incorporation.

Therefore, over the 90 min incubation period rat mast cell histidine decarboxylase was capable of converting virtually all the incorporated histidine to histamine. Although working at the same rate, and therefore forming approximately the same

amount of histamine, the basophil enzyme decarboxylated a smaller percentage of the incorporated histidine since the substrate concentration was higher. If a longer incubation time, without additional substrate being incorporated, was employed the basophils may be capable of converting more of the histidine to histamine.

One million basophils contained 1.13 μg of histamine (Table IX). Over a 90 min incubation when the extracellular histidine and histamine concentration was 1.8 $\mu.moles.l^{-1}$ the same number of basophils synthesized 4.2 ng histamine and incorporated 0.65 ng preformed histamine, 0.4 and 0.06% of the extractable endogenous histamine, respectively.

Since the mast cells contained about ten times more histamine than the basophils, the uptake and synthesis of histamine, under the conditions used, would have little effect on the intracellular histamine concentration. Similar values were obtained by Cabut and Haegermark (1966 and 1968) for rat mast cells. Using essentially the same conditions, in 60 min, the uptake of histamine accounted for 0.02% of the histamine content whereas the formation of histamine from histidine increased the histamine concentration by only 0.015%. They calculated, using the data for mature cells, that a mast cell surrounded by 2 μ $moles.l^{-1}$ histidine would take 30 weeks to build up a histamine store of 15 μg per 10^6 cells.

For the basophil-enriched cell suspensions, if the extracellular histamine concentration were raised to 1 $m.mole.l^{-1}$, the amount of histamine taken up in 90 min would account for about 10% of the intracellular histamine. It was only possible to measure histamine synthesis up to an external histidine

concentration of 100 μ.moles.l^{-1}. At this concentration, over 90 min, the internal histamine concentration was raised 2.2%.

Therefore, using data obtained from a normal resting basophil, it would take about 3 days to produce the amount of histamine present within the granules if the extracellular histidine concentration was 100 μ.moles.l^{-1}. The *in vivo* extracellular histidine concentration is not known but the maturing basophil may be much more efficient at taking up histidine and forming new histamine than the cells used in these experiments. They may take up histidine at a greater rate and if there was very little histamine present within their granules they could decarboxylate the histidine more rapidly.

After an anaphylactic reaction the concentration of histamine in the microenvironment around a basophil or mast cell may be extremely high. In the rat mast cell the histamine concentration has been calculated to be approximately 0.3 moles.l^{-1} (Uvnas, 1969). Consequently, after degranulation the uptake of histamine could be significant in the regranulation process.

Therefore, under normal circumstances, histidine may be the principal source of granular histamine but in certain conditions, e.g. following degranulation, exogenous histamine could replenish part of the stored amine. However, *in vitro* experiments on enzyme activity inform only about the potential rates of histamine formation and histidine and histamine uptake, not about the rate of formation in the living organism. This is because neither the enzyme nor the substrate quantities, not to mention the interrelationships which may exist among various substrates, coenzymes and enzymes, are known.

The transport of most neutral amino acids in animal cells appears to be predominantly mediated, presumably involving a mobile carrier that binds its substrate before translocating it across the "osmotic barrier". This is inferred from the criteria considered characteristic of such transport processes as saturation kinetics, specificity and typical interactions between different amino acids during transport.

The transport of amino acids shows saturation kinetics that often fit the Michaelis-Menten relationship, double reciprocal plots of initial transport rate against extracellular concentration being linear, at least at low concentrations (Heinz, 1954). At high concentrations there is very often deviation from linearity, the line diverging towards the origin. This can be attributed to a non-saturable transport component, often taken as free diffusion which may not always be justified, because a mediated system with sufficiently low affinity, i.e. high K_M, would behave similarly (Christensen and Liang, 1966a).

The kinetics of histidine uptake were studied to determine if there were differences in the transport process of various cell types. The initial uptake of histidine was measured over a wide concentration range, 0.18-1000 μ.moles.l^{-1} (Figs. 9 and 9A). At all concentrations the greatest uptake occurred in the basophil-enriched cell suspensions. Reciprocal plots of initial rate against substrate concentration were not linear and, therefore, more detailed analysis was carried out using a computer programme to compare the experimentally derived data with that predicted by the

Michaelis-Menten relationship and the Hill equation. The experimentally derived values for the uptake of histidine by basophil- and eosinophil-enriched cell suspensions did not fit a rectangular hyperbole as predicted by the Michaelis-Menten equation (Table IV). The observed values were greater than would be expected at low concentrations and lower than expected at high concentrations. This is suggestive of negative co-operativity (Conway and Koshland, 1968; Newsholm and Start, 1973). To test this hypothesis the results for all the cell types were fitted to the Hill equation (Tables V and VI). Negative co-operativity was occurring in the uptake of histidine by all cell types since the Hill coefficients, h, were less than one (Table VII).

Negative co-operativity would arise if the measurements were not initial velocities and/or if there were more than one uptake process each with a different K_M. The values for the maximum velocities, V_{MAX}, and substrate concentrations which give half maximum velocity, s_{50}, are empirical figures but some conclusions can be drawn from them. By comparing the two bone marrow derived cell suspensions the basophils seem to have an effect on the derived values. The V_{MAX} of the basophil containing cell suspension was higher and the s_{50} lower, suggesting that the basophils are more efficient in taking up histidine at low substrate concentrations and, if the substrate concentration were high, they would incorporate histidine to a greater extent.

The transport of a given metabolite by mammalian cells is a complex process and a given transport system can exhibit wide scope, transporting a number of similar substances. Also, a metabolite may simultaneously be transported by two

or more apparently independent systems. The decoding of the several systems involved in the transport of a single metabolite may be quite complicated. A given metabolite may share a transport route with several other metabolites. This may involve two or more transport systems and the degree of sharing may be different for each system and for each pair of metabolites. Therefore, a complex, quantitative inhibition analysis, requiring experimental modification of the environmental conditions is necessary (Christensen, 1966 and 1969). By application of these discriminating techniques, a number of systems for the transport of amino acids have been described (Christensen, 1977). Support for the transport systems which have been described so far come from differences in developmental history of the systems and also from differences in their genetic determination, inducability, repressibility and sensitivity to hormonal stimulation (Christensen, 1973).

The transport systems for neutral amino acids have been defined by using Ehrlich ascites tumour cells, but they are taken to be representative of those in the cells of higher animals in general (Oxender et al, 1977).

The most conspicuous transport systems are the Na^+-dependent A systems and the Na^+-independent L system (Oxender and Christensen, 1963). Essentially all the neutral amino acids are transported to some degree by both these systems. Amino acids with short, polar or linear side chains tend to be preferred by the A systems, e.g. alanine, glycine and serine, while the L system shows preference for branched chain and aromatic amino acids, e.g. leucine, valine and phenylalanine.

Another Na^+-dependent system almost completely specific to glycine is not seen in the Ehrlich cells. It has been defined from its description in the pigeon red blood cells by Vidaver (1964) and is present in nucleated and reticulated red blood cells. A variant form with broader specificity may occur in the intestine and kidney (Christensen, 1975).

A third Na^+-dependent system called ASC has an intermediate range of specificity. The amino acids transported by the ASC system have a 3 to 5 carbon straight chain, their hydroxyl and sulphydryl derivatives and also asparagine, glutamine and the prolines can be incorporated by this route. The ASC system is found in almost every cell type including immature and eunucleated red cells (Wise, 1976a), leukaemic cells (Thomas and Christensen, 1971) and lymphocytes (Wise, 1976b) and it also participates in placental transport (Enders et al, 1976).

Cationic amino acids are transported by a single system, Ly^+. This system requires a positively charged group in the side chain with a pK_{2a} greater than 8.7 (Christensen and Liang, 1966b). Diamino acids may be transported by the neutral systems to a variable degree.

A complete inhibition and kinetic analysis of histidine transport by basophil-rich cell suspensions was outwith the scope and aims of this thesis. However, a few preliminary experiments were carried out to determine if the observed patterns of inhibition obtained were compatible with the already established transport systems for amino acids.

By observations in Ehrlich ascites tumour cells and various red blood cells, histidine is thought to react

equally with the A and L systems (Oxender and Christensen, 1963). It does not seem to be incorporated to any extent by the ASC system (Christensen et al, 1967). An extensive study by Christensen (1968) into histidine uptake by Ehrlich cells, at a concentration of 1 m.mole.l^{-1} at pH 7.4, suggested that 30% of the histidine was taken up by system A, 66% by system L, 1% by system Ly^{+} and 3% by a non-saturable route. This author also postulated that at lower concentrations the dominance of the L system would be greater.

Phenylalanine, leucine and isoleucine are thought to be predominantly incorporated by the L system. Therefore, they should readily inhibit the uptake of amino acids transported by that system but only minimally suppress the uptake by A and ASC systems. Figure 12 shows that the uptake of histidine was depressed by about 60% in the presence of these amino acids at 1 m.mole.l^{-1}.

Tryptophan, tyrosine and methionine divide their uptake almost equally between the A and L systems. The first two mentioned are grouped together with histidine as having approximately the same reactivities towards the two systems (Christensen, 1977). Cysteine can probably be incorporated by all neutral amino acid transporting systems although it is preferentially incorporated by the ASC system which has not been shown to facilitate histidine incorporation to any extent (Christensen et al, 1967). The uptake of histidine by the basophil-enriched cell suspensions was markedly decreased by tryptophan, tyrosine and cysteine and to a lesser extent by methionine (Fig. 11). Cysteine reacts with pyridoxal 5'-phosphate to form thiazolidine derivatives (Buell and Hansen, 1960). It is, therefore, possible that

its primary effect was on histamine formation and that the decrease of histidine uptake could have been due to a negative feedback by histidine. However, the effects of tryptophan, tyrosine and methionine were probably due to direct competition for uptake.

Valine shows a greater reactivity to the L system whereas alanine, serine and threonine are incorporated more readily by the A system. The uptake of histidine was significantly inhibited by valine (Fig. 14) but alanine, serine and threonine were without effect (Fig. 13). Proline which has very little or no reactivity with the L system, and glutamine which reacts equally with the A, L and ASC systems caused no alteration in the amount of histidine incorporated.

Lysine is a substrate for the cationic transport system, Ly^+, but can also be transported to a lesser extent and depending on the pH by the neutral system. The slight inhibition of histidine transport by lysine (Fig. 14) may have been due to the interaction of this amino acid with a neutral system. It is also possible that histidine is a substrate for the Ly^+ system and the observed effect was a measure of histidine uptake by the cationic transport system since at pH 7.4 about 5% of the histidine is present in a cationic form.

A partial requirement for sodium ions was observed since the uptake of histidine was reduced by about 18% in Na^+-free medium (Fig. 19) suggesting that an Na^+-dependent system is involved, at least to a small extent. The role of sodium will be discussed in detail later.

An effect, by the various amino acids used, on the amount of histamine formed de novo could be merely a reflection on the decreased uptake and/or a direct effect on histidine decarboxylase. Histidine is decarboxylated to histamine by two enzymes. A general aromatic-L-amino acid decarboxylase which will decarboxylate histidine, tryptophan, tyrosine, phenylaline, 3,4-dihydroxyphenylalanine (DOPA) and 5'-hydroxytryptophan (5-HTP) has a low activity towards histidine (Lovenberg et al, 1962). There is also a specific histidine decarboxylase which has a high affinity for histidine and does not decarboxylate DOPA or 5-HTP. This enzyme has been shown to occur in a variety of tissues including rat peritoneal mast cells (Rothschild and Schayer, 1958) and bone marrow (Håkanson, 1963a). The non-specific enzyme has been described in many animal tissues (Watson, 1956; Mitchell, 1963) and is probably present in almost all tissues, even in those in which a specific histidine decarboxylase occurs (Hagen et al, 1960).

These two enzymes can be differentiated by the use of various inhibitors. Benzene has been shown to increase the activity of aromatic amino acid decarboxylase but to have no effect on or slightly decrease the activity of the specific enzyme (Mackay et al, 1961). Alpha-methyl DOPA is a specific inhibitor of aromatic amino acid decarboxylase (Robinson and Shepherd, 1962; Levine et al, 1965), whereas an α-methylhistidine (Mackay and Shepherd, 1960) and brocresine (Reid and Shepherd, 1963) affect the specific enzyme.

Galli et al (1976) showed that various inhibitors of histidine decarboxylase reduced histamine synthesis in suspensions of guinea pig blood cells rich in basophils, whereas α-methyl DOPA was without effect. In the present study it is probable that the histidine decarboxylase activity in the basophil-enriched cell suspensions can be attributed to the specific enzyme. Tyrosine, tryptophan and phenylalanine which are substrates for the general aromatic amino acid decarboxylase and would, therefore, be expected to compete with histidine, did not produce any greater inhibition of histamine formation than methionine and cysteine which are not decarboxylated. However, complete characterization of the enzyme(s) present within guinea pig basophils would require the study of the effect of the various known inhibitors in a cell-free system.

Pyridoxal 5'-phosphate under certain conditions may react in an irreversible fashion with amines and amino acids. In such reactions Schiff's bases are transformed into cyclic compounds (see Introduction, Section I, part 5.1). Various complex combinations may occur, depending on the reacting amino acid, possibly leading to the inhibition of decarboxylation processes. Schott and Clark (1952) showed that DOPA decarboxylase was inhibited by histamine and histidine and Mackay and Shepherd (1962) studied the inhibition of histidine decarboxylase by imidazole derivatives.

All the amino acids which produced an inhibition of histidine uptake caused an even greater percentage reduction in the amount of histamine formed. This was most marked with methionine (Fig. 11). The percentage decrease in

histamine formation, by methionine, was approximately the same as was found for tryptophan and tyrosine although it was only half as effective at decreasing histidine uptake.

Alanine, serine and threonine was almost without effect on histidine uptake but gave significant inhibition of histamine formation. Glutamine which had no influence on histidine uptake, decreased the amount of histamine formed by 60% at 1 m.mole.l^{-1} (Fig. 14). Since these amino acids were without effect on histidine uptake it is probable that they inhibited histidine decarboxylase by complexing with pyridoxal 5'-phosphate.

Histidine uptake was decreased when the incubation medium was free of Na^+-ions but the amount of histamine formed was not significantly different from the controls. Therefore, it is probable that the L system, if required, is capable of providing the basophil with virtually all its histidine and at other times with a substantial part of it, the rest being made up by the A and Ly^+ systems and possibly by non-mediated transport.

However, it is worth re-emphasizing that the in vitro techniques used were performed under defined experimental conditions and that in vivo the relative importance of the transport systems may be different.

Stereospecificity, which may be absolute with certain enzyme systems, is less pronounced in amino acid transport. In most cases the affinity of a given system for the L-configuration is several times higher than for the D-configuration, but D-amino acids may be accepted and incorporated by the same system (Christensen et al, 1952).

The uptake of histidine by basophil-rich cell suspensions appears to be confined to the L-stereoisomer. Conclusive evidence could be provided by showing that (i) D-histidine was not taken up by the cells and (ii) the stereoisomers were not mutually inhibitory. In the present study it has been shown that high concentrations of D-histidine had no effect on the uptake of its L-stereoisomer (Fig. 10) and it, therefore, seems likely that the transport system(s) being studied act virtually exclusively on the L-stereoisomer; although the possibility still exists that the D-histidine can enter the cells by a different process.

There was a statistically significant decrease in the amount of histamine formed by the basophil-enriched cell suspensions in the presence of 10^{-3} moles.l^{-1} D-histidine (Fig. 10). Mackay and Shepherd (1962) have shown that D-histidine is capable of forming cyclic compounds with pyridoxal 5'-phosphate. Therefore, the observed inhibitory effect is probably due to inactivation of the coenzyme. Alternatively, the decreased histamine formation could be the result of a small amount of the L-configuration being present in the D-histidine. This, however, seems unlikely since the presence of contaminating L-histidine would have produced an inhibition of histidine uptake.

The energy requirements for histidine and histamine uptake and histamine formation were investigated using various metabolic inhibitors. Whereas the principal mode of action for all the agents used has been established, the possibility that they may have other, as yet undetermined, effects must be considered. Therefore, as with all studies using metabolic inhibitors, the results must be interpreted with caution.

Iodoacetamide, an agent which affects all enzymes with essential sulphydryl groups (and could, therefore, influence a number of metabolic pathways including glycolysis (Webb, 1966; Park et al, 1961)), had a pronounced, although not complete, inhibitory effect on histidine uptake.

2-deoxyglucose and sodium fluoride caused a marked reduction in the amount of histidine incorporated. These agents are well established inhibitors of glycolysis. 2-deoxyglucose is converted to 2-deoxyglucose-6-phosphate which is a competitive inhibitor of phosphoglucoisomerase (Wick et al, 1957). Fluoride ions are known to inhibit enzymes which require Mg^{2+}, Ca^{2+} or similar ions (Peters et al, 1964). Sodium fluoride abolishes the enolase reaction which results in the accumulation of phosphoglyceric acid, presumably because of the formation of magnesium fluorophosphate which binds tightly to the enzyme.

The uncoupler of oxidative phosphorylation, 2,4-dinitrophenol (Loomis and Lipmann, 1948), and the respiratory chain inhibitor, antimycin A (Rieske and Zaugg, 1962), inhibited the incorporation of histidine, indicating that aerobic respiration is required for maximal histidine uptake.

Since contractile proteins, such as microtubules and microfilaments are known to participate in certain secretory processes (Wright and Malanista, 1973; Wessels et al, 1971) it is possible that they may also be involved in the uptake process. They could theoretically transport histidine, preformed histamine or newly synthesized histamine to the basophil granule or they may stabilize the membrane transport systems or enzymes such as Na^{+}-K^{+} dependent adenosinetriphosphatase (Na^{+}-K^{+} dependent ATPase) which are known to facilitate

membrane transport. Cytochalasin B inhibited histidine uptake at the highest concentration used. Although this agent binds to microfilaments, stabilizing them as the protomer (Miranda et al, 1974), and alters membrane-associated functions such as directional migration, pinocytosis and phagocytosis (Allison et al, 1971), it also inhibits hexose transport at doses similar to those used (Cohn et al, 1972). Therefore, its effect on histidine transport may have been due to a decrease in the energy available from glycolysis. No effect was observed with colchicine and, therefore, functional microtubules (Wilson et al, 1974) do not appear to be involved in histidine uptake.

Whether or not the amount of histidine incorporated was affected by the above agents, the amount of histamine formed in the presence of the inhibitors was not significantly different from controls.

The histidine incorporated by the basophils could be used for protein synthesis, in addition to the formation of histamine. Cycloheximide, a recognized inhibitor of protein synthesis (Vazquez, 1974), had a pronounced effect on histidine incorporation but had no effect on the amount of new histamine formed. A similar but less extensive inhibition of histidine uptake, without influencing histamine formation, was found with actinomycin D, an inhibitor of RNA synthesis (Craig, 1973). Mitomycin C which inhibits DNA synthesis (Iyer and Szybalski, 1963) had no significant effect on histidine uptake or histamine formation.

The inhibition observed with actinomycin D was probably a reflection of an effect on protein synthesis similar to

that observed with cycloheximide. This inhibition should, in theory, make more histidine available for histamine formation. There was no increase in the amount of histamine formed in the presence of these inhibitors and, in fact, there was a decrease in the amount of histidine taken into the cells. This suggests that, if blocking protein synthesis did lead to an increase in the intracellular histidine concentration and if under these conditions histidine decarboxylase was saturated, there is a negative feedback by histidine influencing its own uptake.

The cell suspensions, as discussed previously, contained only about 30% basophils. Therefore, the proportion of histidine taken up by the other cell types may vary in the presence of metabolic inhibitors. If these agents affected the different cell types present to a greater or lesser degree, and since histamine formation was not affected by these agents, it was possible that the uptake of histidine by basophils was not influenced by metabolic inhibitors and the decrease in uptake observed could have been due to the almost complete inhibition of uptake in the contaminating cells. However, the pattern of inhibition of histidine uptake in neutrophil-rich, macrophage-rich and normal bone marrow cells (Fig. 21) was very similar to or less than that observed in the basophil-rich cell suspensions. Therefore, the uptake of histidine by the basophils must have been decreased to some extent. This again suggests that under the conditions used histidine decarboxylase was saturated and synthesizing the maximum amount of histamine possible, and that this pathway of histidine catabolism has the greatest affinity for histidine.

Histamine synthesis was virtually completely inhibited by the presence of NSD 1055, an inhibitor of histidine decarboxylase (Mackay and Shepherd, 1960). As in the case of cycloheximide the intracellular concentration of histidine would be expected to increase. The amount of histidine incorporated was significantly reduced in the presence of NSD 1055 again suggesting a negative feedback by histidine controlling its uptake.

There was a slight, although significant, decrease of histamine uptake in the presence of high concentrations of sodium fluoride and antimycin A. The other metabolic inhibitors tested were without effect (Table XI). Since only very small amounts of histamine were incorporated, under the conditions used, there may have been enough endogenous energy present or since none of the agents would inhibit all energy producing processes, enough energy could have been formed by the unaffected pathways to allow histamine transport to proceed unaffected. Alternatively, the uptake of histamine may not require energy directly and the effects of sodium fluoride and antimycin A were due to indirect effects on the membrane or electrochemical gradients.

The transport of solutes may be linked to the simultaneous transport of sodium ions (Mitchell, 1970). This sytem has been used to explain the uptake of sugars (Crane, 1965), amino acids (Kipnis and Parrish, 1965) and noradrenaline (Bogdanski and Brodie, 1969) by various tissues. The energy required for the active transport of sodium ions is provided by the hydrolysis of ATP through Na^+-K^+ dependent ATPase (Skou, 1965). The active transport of sodium ions is specifically inhibited by the cardiac glycoside, ouabain (Allen and Schwartz, 1974). Ouabain inhibited the uptake of

histidine and histamine and histamine formation (Fig. 19). Incubation of basophil-enriched cell suspensions in Na^+-free medium (choline substituted) also significantly inhibited the uptake processes but not histamine synthesis.

As discussed previously part of the histidine uptake may proceed via the Na^+-dependent A system. If there was no extracellular Na^+ ions or if Na^+-K^+-dependent ATPase was inhibited a proportion of the uptake of histidine would be inhibited. The effect of ouabain on histamine synthesis was probably a reflection on decreased cellular activity since the electrochemical gradients would have been affected.

Histamine uptake was severely depressed by incubating the cells in Na^+-free medium but the effect of ouabain was much less pronounced. At pH 7.4 histamine will carry a net positive charge (Kier, 1968) and to keep the electrochemical gradient at the correct potential the movement of Na^+ ions to compensate for this charge would be necessary.

Therefore, the basophil has the capacity to incorporate histidine by an active mediated process and its metabolic requirements are distinct from those required for the synthesis of histamine. In contrast, the uptake of histamine is comparatively small and appears to occur by a passive mediated process.

SECTION II - MODULATION OF HISTIDINE AND HISTAMINE UPTAKE AND HISTAMINE SYNTHESIS BY HISTAMINE, OTHER PHARMACOLOGICAL MEDIATORS AND ANTI-INFLAMMATORY AND ANTI-ALLERGY DRUGS

Histamine was shown to increase significantly the amount of histidine taken up (Fig. 22) and the amount of histamine formed de novo (Fig. 23) in a dose- and time-dependent fashion. At the highest concentrations used ImAA and 1,4-MeImAA also gave a significant enhancement of these two variables. The two other catabolites of histamine tested, N-AcHm and 1,4-MeHm, were without effect.

Recently ImAA has been shown to produce a similar effect to histamine in studies on human and guinea pig eosinophil chemotaxis (Turnbull and Kay, 1976; Jones and Kay, 1977) and human eosinophil complement receptor enhancement (Anwar and Kay, 1978). In all these cases N-AcHm, 1,4-MeHm and 1,4-MeImAA were without effect.

The enhancement of histamine formation followed the same pattern as the increased histidine uptake and may have been due to increased substrate availability for the enzyme histidine decarboxylase. This, however, seems unlikely since, as previously discussed, the enzyme appeared to be saturated at the concentration of histidine present in the control cells and an increase in substrate concentration would have no effect on the amount of histamine formed. In addition, histidine is incorporated by transport systems which also take up other amino acids. A direct primary effect on the transport mechanism for histidine uptake would also influence the potential transport of other amino acids which were not required in greater quantities.

With the concentration of histamine used a substantial amount of histamine may have entered the cells. A direct intracellular effect of histamine, the product of the reaction,

is, however, unlikely to stimulate its production. In fact histamine may, in certain conditions, form a cyclic compound with pyridoxal 5'-phosphate, the histidine decarboxylase coenzyme, resulting in the inhibition of enzyme activity (Rotilio and Mondovi, 1966). Therefore, an increased intracellular histamine concentration would be expected to inhibit histamine formation. Histidine decarboxylase activity can also be controlled by variations in its pH optimum. This mechanism of autoregulation depends on the fact that the available substrate concentration and K_M change with pH (see Introduction). It is possible that a high intracellular histamine concentration may influence histidine decarboxylase activity by affecting the pH in the vicinity of the enzyme.

However, it is unlikely that the observed enhancement of histidine uptake and histamine formation was due to a direct intracellular effect of histamine. The removal of histamine from the external medium, which would probably have no effect on the intracellular concentration of histamine, resulted in the abrogation of the effect (Table XII).

There may be no requirement for histamine to enter the cells since histamine receptors on the plasma membrane when activated by histamine could liberate a secondary messenger into the cytoplasm which in turn could stimulate the uptake of histidine and/or histidine decarboxylase activity. At present, there is no direct evidence that basophils possess such receptors although they have been detected on most other leucocytes (Saxon et al, 1977; Diaz et al, 1979). However, there is indirect evidence for their existence

since the effects of histamine on histamine release (Lichtenstein and Gillespie, 1973) and chemotaxis to C5a (Lett-Brown and Leonard, 1977) were reported to be blocked by H2-receptor antagonists.

None of the histamine receptor antagonists studied produced a significant effect on histidine uptake or histamine formation per se (Figs. 29 and 30). Mepyramine and chlorpheniramine, principally H1-receptor antagonists, caused a significant inhibition of the enhancement of histidine uptake and histamine formation by histamine whereas H2-receptor antagonists, burimamide and metiamide, were without effect. It is, therefore, possible that histamine may act through an H1-receptor to produce increased histidine incorporation and histamine formation.

An increase in cellular cyclic AMP has been suggested as the mechanism for the self-induced inhibition of histamine release (Bourne et al, 1971a). However, only chemotaxis of human basophils to C5a and not to lymphokines or formylated peptides was inhibited by histamine (Lett-Brown and Leonard, 1977). Therefore, a role for cAMP in this case is unlikely since it would be required to depress chemotaxis to C5a but have no effect on the response to the other chemotactic factors.

The interaction of histamine with a receptor would by analogy with receptors for peptide hormones (Sutherland et al, 1965) produce a secondary messenger within the cell which would by itself or by stimulating other systems produce the observed effects. The enhancement of histamine formation could occur by increasing the activity of histidine decarboxylase. This, in turn, would lead to an increase of histidine incorporation since its intracellular concentration would have fallen.

Under certain conditions various stimuli induce histidine decarboxylase activation (Schayer, 1962; Schayer et al, 1959; Assem et al, 1972). The inducible enzyme has a high affinity for histidine and is strongly inhibited by alpha-methyl-histidine but only slightly by alpha-methyl-DOPA, suggesting that it is a specific histidine decarboxylase. Control of the levels of enzyme activity depends on the rate of its synthesis and degradation (Schmike et al, 1965). Certain environmental conditions may stimulate the activation of pre-formed proenzymes and/or an induction of new enzyme formation. However, no covalent activation step is known for histidine decarboxylase. The increase in histidine decarboxylase activity described in many tissues by a variety of stimuli was blocked by inhibitors of protein synthesis (Schayer and Reilly, 1968). Therefore, the synthesis of new protein seems to be required for this activation of histidine decarboxylase.

The formation of a new enzyme would probably take longer than the incubation period employed and significant enhancement of histidine uptake and histamine synthesis was observed within 60 min of the addition of the histamine. Depending on the turnover time of the enzyme stimulation of production and inhibition of breakdown could lead to greater histidine decarboxylase activity. The histamine forming capacity of certain tissues has been shown to increase within 3 hr of stimulation by antigen (Kahlson et al, 1966).

The activity of histidine decarboxylase is also regulated by variations in the optimum pH and the availability of active coenzyme (see General Introduction). If these factors were affected by the product(s) of the interaction of histamine

with a membrane receptor then activation of histidine decarboxylase could result.

As previously discussed, under the conditions used, histamine was incorporated in relatively small amounts compared to histidine. However, the amount taken up can be decreased in a dose-dependent fashion by ImAA, 1,4-MeImAA and 1,4-MeHm probably by simple competitive inhibition (Fig. 23). N-AcHm and L-histidine did not influence histamine uptake. Therefore, there is some degree of specificity in histamine uptake by basophil-enriched cell suspensions.

2-(2-aminoethyl) thiazole, principally an H1-receptor agonist, inhibited histamine uptake in a dose-dependent fashion while the two H2-receptor agonists, "Dimaprit" and 4-methyl-histamine, were without significant effect (Fig. 27). It is, therefore, possible that 2-(2-aminoethyl) thiazole as well as interacting with H1-receptors was also taken up by the same process as histamine into the cells.

It could be argued that a portion of the histamine, associated with the cells, was bound to receptors in the plasma membrane and that the inhibition by 2-(2-aminoethyl) thiazole was a reflection of the amount bound to H1-receptors. However, the H1-receptor antagonists and the H2-receptor agonists and antagonists had no effect on histamine uptake.

Although the H2-receptor agonists did not inhibit histamine uptake the possibility that they are taken into the cells cannot be ruled out. They may enter to a small extent by passive diffusion or by some other mediated pathway distinct from histamine uptake. The same applies to the histamine receptor antagonists.

2-(2-aminoethyl) thiazole significantly inhibited histidine uptake and histamine formation in a dose-dependent fashion. The H2-receptor agonists were much less effective, 4-methylhistamine having no significant effect on histidine uptake. Since it is possible that 2-(2-aminoethyl) thiazole may enter the cells it is also possible that it inhibits histidine decarboxylase. The inhibition could arise through cyclic formation with pyridoxal 5'-phosphate, a mechanism which has been described for amines and amino acids (Mackay and Shepherd, 1962). In this way pyridoxal 5'-phosphate would become inactivated and histidine decarboxylase activity inhibited. It is also possible that it inhibited histidine decarboxylase directly since it is structurally similar to histamine, the product of the reaction. The inhibition of histidine decarboxylase would lead to a decrease in the amount of histamine formed and a build up of histidine which could inhibit its uptake by negative feedback.

The effect of the H2-receptor agonists can also be explained by inactivation of pyridoxal phosphate but not by cyclic compound formation. They could act by a mechanism similar to cysteine (Buell and Hansen, 1960). The interaction with the enzyme at the site where histamine is bound is also possible. The fact that they are not as effective as 2-(2-aminoethyl) thiazole suggests that they are not taken up to the same extent or that they are not as effective at blocking histidine decarboxylase activity.

Since the effect of the histamine agonists on histamine formation was greater than that on histidine uptake, and 2-(2-aminoethyl) thiazole inhibited histamine uptake, it is unlikely that the primary action of these agents was a direct

effect on histidine uptake. By itself a decrease in histidine uptake of about 20% should not cause an even greater effect on histamine formation. As previously shown using metabolic inhibitors, a decrease in uptake of 60% had no effect on the amount of histamine formed. Therefore, the slight effect of 2-(2-aminoethyl) thiazole on histidine uptake must have been accompanied by a primary effect on histidine decarboxylase activity which in turn affects histidine uptake by negative feedback.

Histamine can, therefore, influence its own storage within the basophil. It is possible that basophils possess both types of histamine receptor which, when stimulated, may produce various intracellular effects either concomitantly or separately.

Basophils could be recruited to the site of IgE-mediated histamine release or areas of inflammation and injury by C5a. The high histamine concentration, via H2-receptors, would deactivate the cells from further chemotaxis, holding them at a position where they may be required. The stimulation of the H2-receptor would at the same time inhibit further histamine release until a time when the extracellular concentration had decreased and more was required. Interaction with H1-receptors would be stimulating the waiting basophils to form more histamine and hence be fully armed, or stimulating degranulated basophils to produce new histamine so that they could release again if required.

The pattern of effect of prostaglandins E_1, E_2 and $F_{2\alpha}$ on histidine uptake and histamine formation were similar to those of histamine. The effect on histamine formation was

greater than that on histidine uptake suggesting that the primary effect was an increase in histidine decarboxylase activity which led to increased histamine formation with a concomitant decrease in the intracellular histidine concentration which stimulated histidine uptake.

There was no significant effect on histamine uptake by prostaglandins E_1 and $F_{2\alpha}$. The histamine uptake in the presence of the highest concentration of prostaglandin E_2 was statistically greater than the control but an increase of only 10% was an insignificant effect compared to the histamine content of the cells.

Prostaglandins of the E and F series have been reported to act in opposition to each other. In the rat E prostaglandins cause vasodilation and the F prostaglandins, vasoconstriction (Crunkhorn and Willis, 1971). Prostaglandins E_1 and E_2 have been shown to stimulate adenyl cyclase in human leucocytes (Bourne et al, 1971b; Scott, 1970). E prostaglandins cause inhibition of histamine release from mast cells via a negative feedback mechanism which is probably dependent upon activation of adenyl cyclase via prostaglandin-specific adenyl cyclase receptors (Lichtenstein et al, 1972). In human lung, high concentrations (10^{-4} moles.l^{-1}) of prostaglandins E_1, E_2 and $F_{2\alpha}$ inhibited antigen stimulated histamine release by increasing cAMP levels. However, at low concentrations (5×10^{-8} moles.l^{-1}) there was an enhancement of histamine release with a concomitant fall in cAMP levels (Tauber et al, 1973). Similarly, using human leucocytes Bourne et al (1972) demonstrated that prostaglandins E_1 and E_2 inhibited antigenic histamine release while $F_{1\alpha}$ was ineffective.

Therefore, the fact that prostaglandins E_1, E_2 and $F_{2\alpha}$ stimulated histidine uptake and histamine synthesis suggests that the levels of cAMP may possibly modulate histamine synthesis as well as release.

The human ECF-A tetrapeptides and a synthetic analogue had no effect on histidine and histamine uptake and histamine formation. It is probable that guinea pig ECF-A is chemically distinct from its human counterpart since Jones and Kay (1977) reported that the valyl- and the analogue peptides did not attract guinea pig eosinophils *in vitro* whereas the alanyl-peptide had only slight activity.

5-hydroxytryptamine and bradykinin have not been identified in guinea pig basophils. However, the latter has been shown to increase cyclic nucleotide levels, both cyclic AMP and cyclic GMP, in guinea pig lung (Stoner *et al*, 1973). The effect on cyclic AMP was thought to be secondary to an effect on prostaglandin synthesis; therefore, an *in vivo* effect may be possible where prostaglandins could be formed. 5-hydroxytryptamine has been shown to inhibit histamine release from bovine granulocytes (Holroyde and Eyre, 1976). Neither of these mediators, under the conditions used, influenced the parameters measured.

Therefore, histamine and prostaglandins, which are known to be released from guinea pig cells, were capable of modulating histamine storage by increasing the uptake of histidine and the synthesis of histamine *de novo*. The mode of action of these mediators can only be inferred to occur via changes in cyclic nucleotide levels from their known stimulation of adenyl cyclase (Bourne *et al*, 1974). The

other mediators tested, which were without effect in this system, may influence histamine formation in the species in which they occur naturally or *in vivo* where other biological relationships may be present.

None of the anti-inflammatory drugs nor the anti-allergy agent tested significantly influenced histidine and histamine uptake and histamine formation.

Steroid effects are usually thought to occur through interactions with the genetic material of the cell (Buller and O'Malley, 1976). They affect the appearance of mast cells, especially the granules (Asboe-Hansen, 1952). Whether they cause active degranulation to take place or the reduced granularity is due to inhibited synthesis and regranulation, has not been established. Steroids have also been shown to inhibit prostaglandin release (Lewis and Piper, 1975) but have no direct effect on histamine release, antibody production or antibody-antigen interactions. Therefore, a lack of effect on histidine uptake and histamine formation by resting basophils with a short incubation time is not unexpected.

Non-steroidal anti-inflammatory drugs inhibit antigen and compound 48/80 induced histamine release (Norn, 1971) and prostaglandin synthesis (Vane, 1971). It is thought that non-steroidal anti-inflammatory drugs reduce the ATP content of the cells by uncoupling oxidative phosphorylation, possibly by binding to essential amino groups in the mitochondrial respiratory chain (Whitehouse, 1968). Although the inhibitors of aerobic respiration, 2,4-dinitrophenol and antimycin A (Fig. 17), inhibited histidine uptake the non-steroidal anti-inflammatory drugs tested had no significant effect. The lack of an effect suggests that the reported

influence on ATP levels is not the major factor in the action of these drugs or the guinea pig basophil was not affected to a significant extent. The energy required for histidine uptake is not solely dependent on oxidative phosphorylation and if these drugs did inhibit aerobic respiration to some extent, the energy required for histidine uptake could be supplied by anaerobic pathways or other endogenous sources.

Disodium cromoglycate ("Intal") has been shown by Assem and Mongar (1970) to markedly inhibit antigen stimulated histamine release from the passively sensitized lungs of man and monkey. It also produced a slight inhibition of histamine release from actively sensitized monkey and human leucocytes. However, it had no effect on anaphylactic histamine release from actively or passively sensitized guinea pig lung. This anti-allergy drug has been reported to have no effect on guinea pig PCA reactions (Cox et al, 1970), although it has been shown to inhibit the release of SRS-A from antigen challenged guinea pig lungs. However, the release of prostaglandins was unaffected (Dawson and Tomlinson, 1974).

Intal had no effect on the uptake of histidine or histamine formation which could be due to its inactivity towards guinea pig tissues in general and is probably a true reflection of its lack of effect on the parameters measured.

SECTION III - THE EFFECT OF EOSINOPHILS ON HISTAMINE REPLENISHMENT AFTER AN ANAPHYLACTIC REACTION

Antigen- and polymer-induced histamine release is a non-cytotoxic process. Thus, rat mast cells and human basophils when exposed to sub-optimal doses of compound 48/80 and antigen, respectively, are capable of repeated histamine release (Uvnas and Thon, 1959; Drobis and Siraganian, 1976). The histamine stores in guinea pig skin are replenished approximately 48 hr after a passive cutaneous anaphylactic (PCA) reaction which depleted the histamine by 50% (Jones and Kay, 1976). This repair process could involve regranulation and/or recruitment of new cells into the reaction area.

In the two previous sections the development of a model to study basophil functions, in terms of histamine metabolism, has been described. Having established the basic technique, and shown that the basophil has the capacity to regranulate, histidine uptake and histamine synthesis were studied following antibody-antigen interactions. Several in vitro biochemical modulations of histamine metabolism have been described which may have in vivo implications on histamine formation. In this section the theme of repair after degranulation has been developed by studying the possible interaction of eosinophils, which are present in reaction sites and have been implicated in various aspects of type I hypersensitivity including repair, with basophils.

After actively sensitized basophil-enriched cell suspensions were challenged with antigen there was a significant increase in histamine formation (Figs. 35 and 36). Therefore, the release of histamine and other pharmacological mediators stimulated the synthesis of histamine. This increased synthesis could not be due to the presence of

histamine in the extracellular medium since the cells were washed prior to the incubation with ^{14}C-histidine. The increased histamine formation may have been due to the activation of histidine decarboxylase. This could be a direct effect of removing the end product, activation of pyridoxal phosphate, increasing substrate availability or due to an allosteric effect.

Similar results in terms of increased histamine synthesis have been reported for rat mast cells studied in vivo and in vitro (Schayer et al, 1959; Slorach and Uvnas, 1968), human leucocytes where the antigen was administered in vivo and in vitro (Assem et al, 1972) and in various tissues of sensitized rats and guinea pigs after injection of antigen (Kahlson et al, 1966). However, by measuring the total histamine content of cultured human basophils Drobis and Siraganian (1976) were unable to demonstrate any net synthesis of histamine. This was probably due to the lack of sensitivity of their assay. In the cases where increased histamine formation was reported the possibility that a portion of the increase was due to the presence of extracellular histamine cannot be ruled out.

Eosinophils have been postulated to dampen and control type I hypersensitivity reactions by producing substances which inhibit mediator release, by inactivating the released mediators and by inhibiting mediator replenishment (see Aims). Their role in mediator replenishment was further investigated by studying their effect on histidine uptake and histamine formation by basophils. As discussed above, there was an increase in histidine incorporation and histamine formation following antigen-mediated histamine release. When

eosinophils were incubated with the basophils they did not influence this effect.

An effect of eosinophils on histamine replenishment was postulated after it was found that the removal of eosinophils in vivo with anti-eosinophil serum reduced the time required to restore histamine levels after PCA reaction from 48 hr to 9 hr (Jones and Kay, 1976). However, the experiments carried out in vitro showed that the eosinophil had no effect on the challenged basophils repair processes. Although not conclusively shown, since no in vitro system can simulate the complex interactions which occur within an organism, the results suggest that other factors are involved. These may include the interaction of eosinophils with other cells, mediators or other reaction products. It is also possible that basophils have distinct differences from mast cells in terms of histamine replenishment.

Eosinophils are capable of removing histamine from the site of an allergic or inflammatory reaction. They contain histaminase and have been shown to have other antihistamine properties (see Aims). It was demonstrated in Section II of this chapter that histamine was capable of stimulating histamine formation by the basophil. If the released histamine was removed by the eosinophils the repair process could be curtailed. The basophil was also shown (Section I) to incorporate small amounts of preformed histamine. However, if the concentration of histamine in the extracellular fluid was increased, as after an anaphylactic reaction, a substantial amount of histamine could be incorporated. Again the eosinophil could be responsible

for the removal of this potential source of the amine.

However, Jones and Kay (1976) measured total skin histamine which should include extracellular as well as intracellular histamine. This value was decreased by 55% 1 hr after challenge and the presence or absence of eosinophils had no effect. The amount they measured was presumably mostly within mast cells; therefore, replenishment by the uptake of preformed histamine must have been minimal.

The repair process must, therefore, involve histamine synthesis from incorporated histidine which has been shown to be stimulated after mediator release. Some factor associated with the eosinophil is responsible for slowing down this process. This factor may be present within the eosinophil or the eosinophil may remove a messenger which would otherwise stimulate repair.

Over 50% of the guinea pig eosinophil granule's protein is composed of basic protein (Gleich et al, 1974). Similar arginine-rich cationic proteins have been isolated from the granules of human leukaemic myeloid cells (Olsson et al, 1977). The purified human protein and a crude preparation of guinea pig eosinophil granules had no significant effect on the uptake of histidine and histamine formation by basophil-enriched cell suspensions.

Therefore, the interaction between eosinophils and basophils or mast cells which slows down the repair process appears to be complex and possibly only occurs in vivo or involves only mast cells.

Anaphylactic ^{14}C-histidine and ^{14}C-histamine release

The ^{14}C-histamine which had been formed from the incorporated ^{14}C-histidine was stored in a form which could be released by an antigen-induced antibody-mediated reaction (Fig. 45). Similar histamine release was demonstrated by Galli et al (1976) using concanavalin A and in studies of histidine uptake by rat mast cells (Cabut and Haegermark, 1968) using compound 48/80 as the releasing agent.

Incubation of sensitized basophils with antigen also resulted in a dose-dependent release of ^{14}C-histidine (Fig. 46). The release of histidine could be measured in normal cells and cells incubated in the presence of NSD 1055 which inhibited the conversion to histamine completely. The physiological significance of this release is not clear but it is possible that the released histidine could be converted to histamine outwith the basophils or mast cells and thus spread the effect of an anaphylactic reaction. Cabut and Haegermark (1968) showed that in the presence of NSD 1055 none of the histidine taken up by rat mast cells was associated with the granules. Therefore, the possibility that the release of histidine was due to enhanced spontaneous release or increased leakiness in the presence of antigen cannot be ruled out. However, this seems unlikely since K^+ ions and enzyme markers are not released during a type I hypersensitivity reaction (Johnson and Moran, 1969). The histidine may be loosely associated with the granules and, therefore, easily detached during the differential centrifugation procedure. Until the precise location and amounts of histidine present within the basophils are known the physiological significance of histidine release must remain uncertain.

SECTION IV - GENERAL CONCLUSIONS AND SUGGESTIONS FOR FURTHER STUDY

Guinea pig bone marrow cell suspensions rich in basophils have proved to be very useful for the study of histamine storage and anabolism. The uptake of ^{14}C-histidine was a reliable monitor of basophil functions associated with histamine storage. However, all the cell types studied were capable of incorporating histidine to some extent and the histidine taken up by basophils is available for protein synthesis in addition to histamine formation. Therefore, determination of the amount of histamine synthesized by the action of histidine decarboxylase may be a more accurate gauge of basophil activity since it is the only cell present in normal bone marrow capable of decarboxylating histidine.

The general model required the purification of basophil leucocytes from the bone marrow of antigen-stimulated guinea pigs. The method of Dvorak et al (1974) provided large numbers of basophils although the purity rarely exceeded 40%. Whole sheep's blood was far superior to ovalbumin in consistently stimulating basophil production.

Although histamine is not the only pharmacologically active mediator present within the basophil, it has by far the simplest structure and is the easiest to measure. Therefore, as set out in Chapter II, it was the logical choice for this study.

Basophils appear to incorporate histidine by active mediated transport whereas, although histamine uptake appears to be mediated, it is passive in nature. The uptake of these two compounds and leucine was studied in a number of cell types and in all cases the uptake was greatest in the cell populations containing basophils. This, along with

the results of the kinetic study which was carried out, suggests that the basophil has a greater capacity for incorporating histidine, and possibly other amino acids, than other cell types. Whether this is due to the kinetics of the transport systems or the proportion of the histidine which is incorporated by the different systems proposed for amino acid uptake, is uncertain. Until completely pure basophil suspensions can be obtained, conclusions concerning the efficiency of the basophil in incorporating histidine must remain speculative. Even then, since the histidine is involved in a number of metabolic processes within the cell, conclusive verdicts will probably not be possible until the structure(s) responsible for histidine transport can be studied in cell-free systems.

The majority of the studies carried out were over short incubation periods; therefore, the cells were maintained in a simple balanced salt solution. Tissue culture medium has been used to maintain basophils for up to 72 hr (Galli et al, 1976; Drobis and Siraganian, 1976). However, this adds the complication that histidine uptake and histamine formation are greatly reduced in such a system due to the presence of other amino acids. Most culture systems also employ homologous serum which also has an inhibitory effect (personal observation). This type of problem might be alleviated if histidine with a greater specific activity became available. It would then be possible to measure histidine uptake and histamine synthesis in cultured basophils which would then be in a more physiological medium.

Basophils are end-cells, they are not capable of division, but the present observations and the work of

Galli et al (1976) has established that they retain the enzymes required for the replenishment of their histamine stores and are even capable of increasing their histamine forming capacity after anaphylaxis. However, although they are a good model for studying initial repair after a type I hypersensitivity reaction, they are not the only one.

Mast cells are capable of incorporating histidine and histamine and synthesizing histamine. Mast cell suspensions have been obtained by enzymatic digestion of the surrounding tissue (Pearce et al, 1977). Results of metabolic studies with such cells would have to be interpreted with caution since this harsh treatment may affect the integrity of the cell membrane and produce alterations in their transport systems. The peritoneal cavity of the rat contains free mast cells which have been used in this present study and by others (Cabut and Haegermark, 1968) to investigate histamine formation.

The rat mast cell responds differently in various aspects of type I hypersensitivity to man and guinea pigs. This, along with the fact that although very pure populations of mast cells can be obtained they are not present in very large numbers, makes large scale experiments of the kind used in this thesis impossible. However, they can be induced to release their stored mediators by a variety of stimulants. Degranulation can be initiated by antigen, by antibody in reverse-type reactions, by various macromolecules including compound 48/80 and dextran and by calcium ionophores. The effect of histamine release induced by various stimuli on histamine metabolism could, therefore, be studied using rat peritoneal mast cells.

Mast cells are present in almost every tissue of the body. Those of the skin, respiratory and alimentary tracts and eyes are most commonly involved in type I hypersensitivity reactions. Differences in histidine uptake and histamine synthesis by different tissues under various conditions could be studied in tissue slices. The effect of histamine release could possibly be studied over long periods since the cells would probably remain viable longer. Guinea pig lung can be actively and passively sensitized and this model may prove as useful as the basophil.

The exact location of the incorporated histidine and histamine and the newly synthesized histamine has not been investigated although in the mast cell the incorporated histidine was not associated with the granules (Cabut and Haegermark, 1968). The ^{14}C-histamine synthesized from the incorporated ^{14}C-histidine was released by antigen challenge along with endogenous histamine. Therefore, it seems likely that the newly formed histamine is stored in the granules. The amounts of ^{14}C-histamine taken up were insufficient to measure release of radioactively labelled histamine. However, if larger substrate concentrations were employed this may be possible. A certain amount of labelled histidine was released along with the histamine by antigen challenge. Further investigations are required to ascertain whether this is of physiological importance. The intracellular concentration and location of histidine and the amounts released by normal basophils during an anaphylactic reaction will have to be determined.

By differential centrifugation the exact location of the radioisotope could possibly be identified. Autoradio-

graphy may provide another clue to the location and would confirm that the major cell type involved in histidine and histamine uptake in the cell suspension used was the basophil.

Over 20 years ago Sutherland and Rall (1958) identified adenosine-3',5'-monophosphate (cAMP) as the intracellular "second messenger" mediating adrenalin stimulation of hepartic glycogenolysis. Since that time cAMP has been implicated in the control of processes in fields as varied as endocrinology, immunology, neurophysiology, microbiology, plant biochemistry and cell growth and differentiation (Jost and Rickenberg, 1971; Pastan et al, 1975).

Vasoactive hormones and mediators of inflammation have been shown to modulate immune processes through cAMP and possibly cGMP levels (see General Introduction).

Histamine and prostaglandins which have been shown to increase cAMP caused an increase in histamine formation and a concomitant increased histidine uptake. However, the precise role of cAMP in the enhancement of histamine formation is uncertain at present. An extensive study, using agents such as β- and α-adrenergic agonists and metabolic inhibitors which affect the intracellular levels of cAMP as well as measuring variations in cAMP concentrations, would be necessary before firm conclusions could be drawn.

The effect of histamine was abrogated by H1-receptor antagonists suggesting that the basophil membrane possesses receptors for this amine. Other effects of histamine on the basophil have been assigned to stimulation of H2-receptors (Lett-Brown and Leonard, 1977; Lichtenstein and Gillespie, 1973). Histamine receptors can be visualized by the rosette

technique which has been used to identify their presence on a number of different cell types (Diaz et al, 1979; Saxon et al, 1977). It would, therefore, be of interest to determine if the postulated histamine receptors can be demonstrated unequivocally on the basophil plasma membrane. This would give more weight to the theory that the enhancement of histamine formation occurring via changes in cAMP levels.

The histamine and prostaglandins used to demonstrate the enhancement of histidine uptake and histamine formation were synthetic in origin. Authentic mediators can be obtained from actively and passively sensitized guinea pig lungs by challenge with the appropriate antigen (Kay et al, 1971). These so-called anaphylactic diffusates could then be used to determine if they influenced histamine metabolism by the basophil. They would contain several other pharmacologically active mediators including SRS-A, PAF and ECF-A which would all be from the same species as the basophils on which they were being tested. Such a system might mimic in vivo conditions more closely.

The initial aim of the work described in this thesis was to investigate the effects of eosinophils on histamine replenishment following an anaphylactic reaction, using the guinea pig basophil as a single cell model.

Following an anaphylactic reaction, basophils were still capable of forming new histamine and indeed there was a stimulation of histamine synthesis. However, the eosinophil was without effect. This may have been due to a variety of reasons.

The basophil may not be a good model for investigating repair after anaphylaxis since it is turned over fairly rapidly and is an end cell.

Secondly, for technical reasons, the ratio of eosinophils to basophils used was low, ranging from as little as 2:1 to 5:1. The eosinophil comprises about 1-4% of the blood leucocytes in the guinea pig. However, cell suspensions containing a greater proportion of eosinophils can be obtained from the peritoneal cavity of animals stimulated by multiple injections of horse serum (Kay, 1970b). Cell suspensions containing up to 40% eosinophils can be obtained by these methods and they are easily purified further by density gradient centrifugation. However, the total number of cells obtained from each guinea pig is very small compared to the number of basophils required for the experiments. Recently a method has been developed which has been reported to give much larger numbers of cells (Pincus, 1978). This method could be used in the future to produce sufficient cells for the investigation of the role of the eosinophil or eosinophil-derived products on histamine replenishment following anaphylaxis.

Thirdly, the mediators which were released had to be removed by washing the cells after the challenge with antigen. The antigen used was a sheep's blood cell lysate produced by exposing the pelletted cells to distilled water. The viability of the basophil-enriched cell suspensions was affected if the cells were left in contact with the antigen for long periods after challenge. The antigen was removed by washing the cells after 15 min and, therefore, the released mediators were also removed. It would be possible to purify

the antigen(s) by removing the haemoglobin and other cellular contents. This purified antigen could also be concentrated and a constant known amount could be used to induce mediator release. Using the purified antigen, at low concentrations, it may be possible to adjust the level so that the cells release a maximum amount of mediators without their viability being affected by contaminants of the antigen preparation. It would then be possible to study the effect of anaphylaxis *per se* and the effect of eosinophils on the repair process in the presence of the released pharmacologically active mediators.

The eosinophil by itself may not be the only controlling factor in the replenishment of mediators after a type I hypersensitivity reaction. It may accomplish its observed effect of slowing down mast cell repair by interacting with other cell types, by removing and modifying substances or be stimulated itself by substances present *in vivo* which were not present in the experimental incubation medium.

The various mediators and drugs were studied on resting basophils. The effect of these agents might have been different if the cells had been challenged with antigen and, therefore, already stimulated to produce more histamine. Either the basophil model using tissue culture medium or possibly tissue slices could be used for this investigation since longer incubation periods would be necessary.

As mentioned previously, guinea pig tissues can be passively sensitized with antibody directed against a specific antigen. It would, therefore, be possible to vary the degree of sensitization and the class of antibody involved.

Preliminary uptake studies using tissue culture conditions were complicated by the fact that, along with certain amino acids, serum inhibited the uptake of histidine and histamine formation. The effect of serum and antibodies of different classes and at different concentrations requires investigation.

The guinea pig basophil can, therefore, be used to study histamine formation under various conditions by measuring histidine and histamine uptake and histamine synthesis; but that is by no means the only area where this model could be used.

Basophils and/or mast cells have been reported to be capable of phagocytosis (Sampson and Archer, 1967), pinocytosis (Chan and Yoffey, 1960) and the production of superoxide radical (Henderson and Kaliner, 1978). They could be a source of many enzymes including histidine decarboxylase, esterases and proteases for kinetic studies. Since they can be obtained in large numbers the presence of other enzymes and mediators and the structure of the known mediators could be investigated. Basophils from the peripheral blood have been used by Galli et al (1978) to produce an anti-basophil serum which may be useful in elucidating basophil function in vivo.

The release of mediators can be stimulated by a number of factors apart from antigen. These include antibody directed against IgE or equivalent, formylated peptides, cytotoxic release following interaction of antibody against surface antigen and complement activation, reagents such as con-canavalin A and calcium ionophore and in patients suffering from cold urticaria by exposure to low temperatures. The

exact mechanism by which these factors induce release could be investigated along with the reasons why certain agents, such as compound 48/80 and disodium cromoglycate, react with the cells from one species and not another.

Rat peritoneal mast cells have been shown to adhere to complement coated parasites, suggesting that the mast cell possesses complement receptors (Sher, 1976). The rosette technique could be used to investigate the presence of complement receptors on guinea pig basophils. Basophils are also capable of migrating towards certain agents including C5a, lymphokines and suspensions from bacterial cultures. The role of chemotaxis in the function of the basophil is not completely understood. They are known to accumulate in skin sites in delayed-type hypersensitivity (see General Introduction). The guinea pig basophil could be used to study the factors which influence this migration. The effect of the chemotactic factors on any complement receptors identified could also be examined since it has been shown that agents which induce chemotaxis are also capable of increasing the number of detectable complement receptors in various cells (Anwar and Kay, 1978; Kay et al, 1979).

It has been suggested that mast cells or their products play a part in the local tissue resistance against the development and growth of tumours (Cramer and Simpson, 1944; Engelbreth-Holm and Asboe-Hansen, 1953). A role for the mast cell in the host response to parasite infection has also been suggested (Mulligan et al, 1965; Ogilvie, 1974). In this case the eosinophil is also implicated (Butterworth et al, 1977; Anwar et al, 1979).

Further investigation of these diseases could involve the use of single cell experimental systems. Therefore, the guinea pig basophil model might prove useful in the unravelling of a number of important medical problems.

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PUBLICATIONS

The following work connected with this thesis has been published.

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